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35 U.S.C §112 First Paragraph Rejections

POSSESSION OF THE BROAD GENUS OF THE MODULATORS OR AGENTS

The Examiner rejected claims 2, 5, 6, 12, 14, 16, 26, 29, 30, 36, 38, 40, 49, 52, 53, 59, 61, 63, 105, 118, 131, 144-155, 158-169 and 172-188 as failing to comply with the written description requirement. The Examiner alleged that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors at the time the application was filed has possession of the claimed invention.

The Examiner stated that the specification only describes the structure of a nucleic acid encoding bacteriocin release factor. The Examiner asserted that the specification does not teach the structure of any other secondary effector molecule except bacteriocin release factor BRP.

The Examiner stated that in analyzing whether the written description requirement is met for genus claims, it is first determined whether a representative number of species have been described by their complete structure. The Examiner stated when the claims are analyzed in light of the specification, instant invention encompasses genus of nucleic acid encoding any secondary effector molecule. However, the Examiner stated that the specification only describes the structure of a nucleic acid encoding bacteriocin release factor. The Examiner stated while the specification discloses that the secondary effector molecule could be any molecule, the specification does not teach the structure of any other secondary effector molecule except bacteriocin release factor BRP.

The Examiner additionally stated that the claimed invention encompasses any nucleic acid encoding any angiogenic factor from any animal. The Examiner stated while the specification provides a laundary [sic; laundry] list of the anti-angiogenic factors in pages 5-8, it does not teach the structure of the anti-angiogenic factors from a representative number of animals.

The Examiner stated that then it is determined whether a representative number of species have been sufficiently described by other relevant identifying characteristics (i.e. other than nucleotide sequence), specific features and functional attributes that would distinguish different members of the claimed genus. The Examiner stated that in the instant case, the only other identifying characteristic is that the secondary effector molecule will provide additional therapeutic value and/or facilitate the release of the contents of the modified bacterial vector (see page 41). The Examiner stated however, the specification does not disclose any identifying characteristic as to how an artisan would have differentiated different members of the claimed genus. The specification dislxoses [sic; discloses] that the secondary effector molecule is proteinaceous or nucleic acid molecule, however the specification does not teach any characteristics of the proteins and nucleic acid encompassed by the claimed genus. Regarding the anti-angiogenic factors, the Examiner stated that the specification does not provide any description as to what were the identifying characteristics of the nucleic acids encoding the anti-angiogenic factors of different animals that would be representative of genus animals which is a large genus which would have subgenuses.

The Examiner stated that accordingly, this limited information is not deemed sufficient to reasonably convey to one skilled in the art that the applicant is in possession of the broad

genus of the modulators or agents at the time the application was filed. Thus it is concluded that the written description requirement is not satisfied for the claimed genus.

In response, Applicants respectfully traverse the above ground of rejection. Applicants maintain that the written description requirements have been fulfilled. Applicants further maintain that to an ordinary skilled artisan, Applicants have provided written description which shows the possession of the claimed invention i.e. broad genus of the modulators and agents.

Applicants have designated a specific section describing the Secondary Effectors. See beginning bottom of page 41 to 47 of the specification. A general description of the characteristics of the Secondary Effectors is provided. See Page 41, first paragraph. More concrete examples are depicted in the text following the general description. For instance, "Antisense nucleotides" are described on page 41, beginning line 25; "Pro-drug converting enzyme" are described on page 43 being line 7 and "Inhibitor of Inducible Nitric Oxide Synthase (NOS) on page 43, line 24 to 28, specification.

Regarding the anti-angiogenic factor, applicants provide sufficient description to show the possession of said claimed invention. Section 14 entitles "EXPRESSION OF ANTI-ANGIOGENIC FACTORS..." page 84-87, line 22-24 Specification. Construction of a plasmid containing the nucleic acid sequence encoding thrombospondin AHR are described in the specification, beginning page 84, line 29 to page 85, line 17. Construction of a plasmid containing the nucleic acid sequence encoding platelet factor-4 peptide is described on page 85 of the Specification. Construction of a plasmid containing the nucleic acid sequence encoding apomigren is described on page

86. In addition, as summarized clearly in the background of the invention, page 5, section 2.5, specification, anti-angiogenic factors were known at the time of the invention. Applicants' claimed invention is expressing these factors in the tumor-targeted bacteria, not the factors themselves.

Accordingly, in view of the forgoing, Applicants maintain the written requirements have been fulfilled and respectfully request the reconsideration and withdrawal of this ground of rejection.

ENABLING REQUIREMENTS

The Examiner rejected claims 2, 5, 6, 12, 14, 16, 105, and 144-155 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for an attenuated *Salmonella* typhimurium wherein said attenuated *Salmonella* comprises a first nucleic acid encoding a primary effector molecule is endostatin and a second nucleic acid encoding a secondary effector molecule wherein said secondary effector molecule is BRP, does not reasonably provide enablement for any attenuated tumor targeted bacteria comprising any number of nucleic acids encoding any number of primary effector molecules and any number of secondary effector molecules. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The Examiner further rejected claims 26, 29, 30, 36, 38, 40, 118 and 158-169 and claims 49, 52, 53, 59, 61, 63, 131 and 172-188 as failing to comply with the enablement requirement.

The Examiner stated that the invention of claims 2,5,6,12,14,16,105, and 144-155 recites an attenuated tumor targeted bacteria that comprises one or more nucleic acid molecules encoding one or more primary effector molecules and one or more secondary effector molecules operable linked to one or more promoters, wherein said attenuated bacteria is a facultative aerobe or facultative anaerobe. Dependant claims recite a list of anti-angiogenic factors. Claims 26, 29, 30, 36, 38, 40, 118, and 158-169 recite a pharmaceutical composition whereas claims 49, 52, 53, 59, 61, 63, 131 and 172-188 recite a method of targeted delivery to tumor.

Attenuated Tumor Targeted Bacteria

The Examiner stated that the specification as filed is not enabling for the claimed invention commensurate with the scope of the claims because the specification does not sufficient guidance to make any attenuated bacteria comprising any number of nucleic acids encoding any number of primary and secondary effector molecules, treatment of a tumor with the bacteria and targeted delivery of the bacteria to a tumor. The Examiner stated that an artisan of skill would have required undue experimentation to make and use the claimed invention commensurate with the full scope of the claims because the art of targeted delivery of gene and treatment of a tumor using attenuated bacteria was unpredictable and was not routine in the art.

The Examiner stated that the specification on page 10 discloses that the genetically engineered *Salmonella* has been demonstrated to be capable of tumor targeting, possesses anti-tumor activity and are useful in delivering effector genes such as HSV TK to solid tumors (see lines 1-4). The specification, on page 31-32 also, discusses

Salmonella and its mutant as vectors. The specification does not teach any other bacteria as vectors for delivery of effector molecules. The Examiner stated that Dietrich et al (Antisense & Nucleic Acid Drug Development 10:391-399, 2000) reviewed the state of the art bacterial system for the delivery of eukaryotic antigen expression vectors and noted that the four bacteria used as delivery systems were *Salmonella typhimurium, Shigella flexmeri, E.coli* and *Listeria monocytogenes*. Of these only *Salmonella, Shigella* and *Listeria* have been used *in vivo* in mice or rat and none of the bacteria have been used in humans (see table 1). The Examiner stated that additionally, none of these were used for tumor targeted delivery, rather they were used vaccination. The Examiner stated that this clearly shows that at the time of the invention tumor targeted delivery of effector molecule *in vivo* in any animal, including humans was not routine and in the absence of teaching in the art of record, the specification has to teach making and using of any attenuated bacteria for effector molecule delivery in any animal including humans. The Examiner stated that the specification fails to provide enabling disclosure for the claimed invention. The Examiner stated that the method of producing an attenuated bacterium for in vivo delivery of effector molecules was unpredictable at the time of the invention because a rational approach to design a live attenuated bacterial vaccine involves genetic modification of the bacterial pathogen to make the pathogen less virulent while maintaining the stability of protective antigen expression that provides immune protection. The Examiner stated that the attenuation should be an inherent property of the bacterial vaccine and not be dependant on fully host defenses and immune response capabilities (Curtiss R. J. Clin. Invest. 110(8):1061-1066, 2002). The Examiner stated the attenuation of a bacterium requires the modification of a specific bacterial gene that render the bacterial strain

non-virulent. The Examiner stated that for example, inactivation of PhoP/phoq regulatory system in S. typhi results in strains, which are suitable attenuated for uses as vaccines. The Examiner further alleged that the development of live attenuated bacterial vaccine has not been always predictable. The Examiner stated for example, development of a live attenuated *Shigella* vaccine that is sufficiently attenuated to be a non-reactive yet adequately invasive to be highly immunogenic took 30 years in making, since it required substantial understanding of molecular genetic basis of virulence of *Shigella* (Curtiss page 1063, col.2). The Examiner stated that the specification fails to disclose what are the bacterial regulatory systems in these bacteria, mutation of which would result in the making of a live attenuated bacterial strain that would provide protect a mammal against any specific bacterial infection. The Examiner stated that the specification does not provide any guidance to make any attenuated bacteria for tumor targeted delivery. The Examiner stated that it is emphasized that the art of record while teaches attenuated *Shigella* and Salmonella for vaccination, does not teach making attenuated bacteria for tumor targeting.

In response, Applicants respectfully traverse the above ground of rejection. Applicants maintain that the claimed invention has been fully enabled by the claimed invention. Applicants would like to point out that the vaccine studies are irrelevant to the applicants' claimed invention.

The requirements for vaccine vectors are quite different from the tumor-targeted vectors of the claimed invention. Vaccine vectors: (1) are intended to elicit an immune response so the bacterial vaccine must be immunogenic; and (2) must not be capable of excessive growth *in vivo*. Vaccine vectors, in many

cases, contain plasmid DNA encoding the immunogen (or antigen) driven by an **eukaryotic** promoter. The vaccine vectors, after being administered to the mammals, have to be lysed in order to release the plasmid DNA, which has to be subsequently taken by mammalian cells in order to produce the immunogen. The Examiner is correct that the process of plasmid transfer from bacteria to mammalian cells is inefficient and unpredictable. In contrast, tumor-targeted bacterial vectors use **prokaryotic** promoters to make effector molecules, such as anti-angiogenic factors. The tumor-targeted bacteria, unlike the vaccine vectors, act as both DNA delivering vector and effector molecule producer and do not require the transfer of their DNA molecule to mammalian cells.

Applicants' claimed invention can be used with any attenuated bacteria. As stated above, vaccine vectors are different from tumor-targeted vectors. Applicants want to point out the successful application of attenuated *Salmonella* in inhibiting tumor growth in mammals: (1) Einstein et al (Medical Oncology 12, 103, 1995) used an AroA- auxotrophic mutant; (2) Pawelek et al (Cancer Res., 57:4537, 1997) used purine auxotrophic mutants; and (3) Luo et al., (Oncol Res., 12:501, 2001), used a msbB-purI- mutant. A copy of these references is attached hereto as **Exhibit A, B and C** respectively.

As recited hereinabove, the Examiner seems to contend that microorganisms other than *Salmonella* in the claimed invention are not enabled. This is not true. The specification provides sufficient teachings to enable one skilled in the art to make and practice the invention for microorganisms other than *Salmonella,* the teachings of the invention being exemplified using *Salmonella* as a model microorganism.

In support of this position, a Declaration by D. David Bermudes with attached **Exhibits 1 and 2** ("the Bermudes Declaration"), one of the inventors of the present application, was submitted in U.S. Patent No. 6,685,935, issued February 3, 2004, U.S. Serial No. 09/358,052, filed July 21, 1999. The Bermudes Declaration, a copy of which is attached hereto as **Exhibit D**, demonstrates the use of *non-Salmonella* microorganisms as taught by the specification. Specifically, the Bermudes Declaration describes experiments that were conducted using the guidelines set forth in the specification, together with basic microbiology techniques known to those of skill in the art. See Bermudes Declaration at paragraphs 5, 7 and 8. The Bermudes Declaration clearly shows the *in vivo* tumor-targeting ability of *non-Salmonella* microorganisms as claimed, namely a gram-negative *Shigella* species and *Listeria monocytogenes*, a gram-positive species, and of a gram-positive *Streptococcus* species. See Bermudes Declaration at paragraph 7, Exhibit 1. These tumor-specific microorganisms, when administered to experimental mice with established melanoma tumors, inhibited tumor growth by approximately 40-65%, without any lethality due to the administration of the microorganisms. See Bermudes Declaration at paragraph 8, Exhibit 2.

Dr. Bermudes has concluded: "it is my considered scientific opinion, that the use of the tumor-specific microorganisms, including but not limited to non-*Salmonella* genera of microorganisms, for reducing volume or inhibiting growth of a tumor, can be practiced according to the methods described in the present specification by one of ordinary skill in the art without undue experimentation". Bermudes Declaration at paragraph 9. Further, Dr. Bermudes affirms: "Based on the experiments described (in my declaration), I conclude, and I believe one of ordinary skill in the art would also conclude, that the teachings of the specification are applicable to *non-*

Salmonella microorganisms. Therefore the specification provides sufficient guidance to identify tumor-specific *non-Salmonella* microorganisms and to administer the tumor-specific *non-Salmonella* microorganisms to a subject to inhibit tumor growth or reduce tumor volume. Accordingly, I conclude, and I believe one of ordinary skill in the art would also conclude, that the claimed invention can be practiced without undue experimentation by following the teachings of the specification". See Bermudes Declaration at paragraph 10. Thus, as stated in the Bermudes Declaration, at paragraphs 5 and 9-10, the specification provides enablement for practicing the claimed invention.

Other groups such as Yu, et al. (2004) discloses expression of genes in *Escherichia coli* and three attenuated pathogens: *Vibrio cholerae, Salmonella typhimurium, and Listeria monocytogenes*, all entered tumors and replicated. A copy of Yu, et al., entitled "Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins" Nature Biotechnology, 22:313-320 is attached hereto as **Exhibit E**.

In addition, Szalay, Aladar A. (2003) discloses expression of endostatin in other bacteria. See particularly, paragraph 106. A copy of Szalay, Aladar A., entitled "Light emitting microorganisms and cells for diagnosis and therapy of tumors" U.S. Patent Application No. 20030059400 is attached hereto as **Exhibit F**.

The above post-filing experiments and publications show clearly that following the teachings provided by the applicants' specification, an ordinary skilled artisan can

practice the claimed invention in microorganisms other than *Salmonella*.

Accordingly, in view of the forgoing, Applicants maintain that the claimed invention has been fully enabled by the filed disclosure and respectfully request the reconsideration and withdrawal of this ground of rejection.

Anti-angiogenic proteins

The Examiner stated that additionally, claims invention recites nucleic acid encoding any anti-angiogenic protein from any animal, however, neither the specification teaches structure of the nucleic acid encoding a representative number of factors nor does it teach how to make and use the nucleic acid and any attenuated bacteria comprising them.

In response, Applicants respectfully traverse the above ground of rejection. Applicants maintain that the specification as filed has provided enough teachings to enable an ordinary skilled artisan to practice the invention. Structures of the nucleic acid encoding anti-angiogenic protein are known at the time of the invention. See page 5, section 2.5 of the specification. Applicants have designated section 14 for the "EXPRESSION OF ANTI-ANGIOGENIC FACTORS BY ATTENUATED TUMOR-TARGETED SALMONELLA" beginning page 84, line 22, Specification. Construction of a plasmid containing the nucleic acid sequence encoding thrombospondin AHR (one of the Anti-angiogenic protein) are described, page 84, line 29 to page 85, line 17. Construction of a plasmid containing the nucleic acid sequence encoding platelet factor-4 (another example of the anti-angiogenic protein) peptide is described on page 85. Construction of a plasmid containing the nucleic acid sequence encoding apomigren (a further example of the

anti-angiogenic protein) is described on page 86 and anti-angiogenic peptides produced by *Salmonella* inhibiting endothelial cell proliferation is described on page 87. Therefore, contrary to the Examiner's allegation, expression of anti-angiogenic factors in microorganisms has been described and enabled in the applicants' disclosure. Accordingly, Applicants respectfully request the reconsideration and withdrawal of this ground of rejection.

***In vivo* - Treatment of Tumor**

The Examiner stated that given the lack of guidance or direction provided by the instant specification into would have required undue experimentation to use the claimed attenuated bacteria for *in vivo* gene delivery and treatment of tumor in any animal including humans. It is emphasized that while there is evidence for vaccination using Salmonella or Sighella there is no evidence for targeted delivery of a nucleic acid encoding an anti-angiogenic protein in a tumor. For bacteria to function as DNA delivery systems into mammalian cells in general or in a human in particular, the bacteria must first enter the cell and then escape from the vacuole to the cytosol. Movement from the vacuole to the cytosol is unpredictable because in many instances the bacteria are lysed by the host cell's defense system and any plasmids carried by the bacteria are degraded preventing expression of heterologous nucleotide sequences. The Examiner stated that at best it would appear that only a few calls, if any may be transformed with plasmid DNA carried by a bacterial vehicle as Grillot-Courvalin (Nature Biotechnology, 1998,16:862-866) suggest that "direct introduction of DNA from bacteria to mammalian cells has been reported in very few instances". See page 865, stating with the first line of the discussion. The Examiner stated that Grillot-Courvalin support such observations by reporting that "factors such as

entry route may have an effect" on DNA delivery. The Examiner stated that Grillot-Courvalin go on to report that a mouse dendritic cell line, which can internalize bacteria via micropinocytosis, did not express incoming DNA at 24 hours post-transfer. The Examiner stated that Grillot-Courvalin suggests that this failure could reflect rapid degradation of the invading bacteria by this cell type. The Examiner stated that it would appear that use of bacteria as DNA delivery vehicles is not very efficient in other cell lines as well as Grillot-Courvalin have reported that *E.coli* carrying a nucleotide sequence encoding the green florescent protein are only able to transform 0.3-1% of transfected macrophage cell line. These observations are corroborated by Dietrich et al (Nature Biotechnology,1998,16: 181-185) who report that only about 0.03% of macrophages infected with a mutated form of *Listeria monocytogenes* express a green fluorescent protein reporter gene. The Examiner stated that Dietrich et al also suggest that expression of a heterologous nucleotide sequence is not stable over time by observing a gradual loss of florescence over time. The Examiner stated that Dietrich report that the low efficiency of expression of GFP as compared to the number of macrophages infected may be due to the fact that "only some of the attenuated bacteria infecting the host cells survive the antimicrobacterial milieu inside the phagosome and are able to escape into the host cell cytosol, whereas the others are totally digested, including the plasmid DNA and that not all listeriae being taken up reach the host cell cytosol as an intact viable entity, but the plasmid DNA is still released into this compartment."

In response, Applicants respectfully traverse the above ground of rejection. The Examiner is corrected that DNA transfer from bacteria to mammalian cells is very inefficient and unpredictable. Applicants would like to point out that the

instant invention, using tumor-targeted bacteria for cancer treatment, **does not** involve DNA transfer between bacterial vectors and mammalian cells. Every bacterium contains the gene of interest and produces the said protein product by the bacteria.

Accordingly, in view of the forgoing, Applicants respectfully request the reconsideration and withdrawal of this ground of rejection.

Route of Administration

The Examiner stated that claimed invention encompasses delivery by administering the bacteria any route, however, it is not routine in the art to administer the bacteria by any route. The Examiner stated that while the art administration by oral route, the issues of unpredictability regarding stability and antigen expression at a level sufficient to induce an immune response abound. The Examiner stated that a general issue of unpredictability of oral vaccines is the poor immunogenicity displayed by most antigens when given orally. See Pascual et al (Behring Inst. Mitt., 1997, 98:143-152) on page 143. The Examiner stated that Pascual et al report that there are several issues compounding the development of live oral bacterial vaccine vectors, including the fact that there is a "lack of a well tolerated, highly immunogenic bacterial vector for use in humans." The Examiner stated that while the instantly claimed invention is not directed to vaccination and is directed to tumor directed delivery, the issues of unpredictability discussed above will be applicable in the instant case.

In response, Applicants respectfully traverse the above ground of rejection. Applicants would reiterate that vaccine studies

are different from the claimed invention. See *supra*. Therefore, the teaching of vaccine studies is irrelevant.

Applicants maintain that different routes of administration have been enabled by the filed specification. Following the teaching of the applicants' disclosure, *Salmonella* carrying a heterologous cytosine deaminase gene (TAPET-CD) has been successful used to deliver and to express the enzyme in tumors. Using the route of intravenous administration, King et al., Human Gene Therapy 13:1255, 2002, showed that cytosine deaminase was expressed in tumors in mice receiving TAPET-CD. A copy of the King et al., (2002) is attached hereto as **Exhibit G**. In a human clinical trial, cytosine deaminase enzymatic activity was detected in tumors intralesionally injected with TAPET-CD, Nemunaitis et al., 10:737, 2003, a copy of which is attached as **Exhibit H**.

Accordingly, in view of the forgoing, Applicants respectfully request the reconsideration and withdrawal of this ground of rejection.

CONCLUSION

Applicants believe that all grounds of objections and rejections raised in the outstanding Office Action have been fully addressed. Accordingly, Applicants respectfully request the reconsideration and withdrawal of these grounds of objections and rejections and respectfully request favorable action to be rendered by the Examiner.

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Communication. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

Albert Wai Kit Chan

I hereby certify that this paper is being deposited this date with the U.S. Postal Service with sufficient postage for first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

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Immunotherapy of a plasmacytoma with attenuated salmonella

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An attenuated strain of *Salmonella typhimurium*, SL3235, developed as a prototypic typhoid vaccine, is shown to retard growth of a murine plasmacytoma, TEPC-183, and to prolong survival of tumor-bearing mice. Live salmonella, but not acetone-killed organisms, had antitumor activity. The immunotherapeutic effect was demonstrable when the tumor was injected intralesionally or intraperitoneally. Increased survival, longer mean time to death, and retardation of tumor growth were found when the salmonella were given intralesionally as late as the sixth day post-tumor injection. Timing of salmonella inoculation, as well as the salmonella dose, had an effect on treatment efficacy. Injection of salmonella intraperitoneally exerted a strong antitumor effect when given as late as the third day post-tumor inoculation. The highest dose (2×10^6) of salmonella was less effective than doses 10- or 100-fold lower. TEPC-183 plasmacytoma is rapidly growing and highly immunosuppressive, so the ability of the salmonella to exert therapeutic activity against it is a measure of the potency of the vaccine. These observations are of interest, as they show that a genetically engineered, avirulent strain of Salmonella has immunotherapeutic properties similar to those of BCG and other biological response modifiers, and might have clinical potential as an antitumor agent.

Keywords: salmonella; plasmacytoma; biological response modifier; immunotherapy

INTRODUCTION

Murine plasmacytomas have been extensively studied as models of tumor-induced humoral immune suppression. TEPC-183, a murine plasmacytoma (IgM), grows rapidly, suppresses production of normal immunoglobulins as well as immune responses to T-dependent and T-independent antigens [1-3], and sensitizes mice to infection [4]. The fact that these tumors are profoundly immunosuppressive may account, in part, for their rapid growth.

We have shown that a live, attenuated strain of *Salmonella typhimurium*, SL3235, is a potent activator of macrophages. When given as a vaccine, it induces tumoricidal macrophages in C3HeB/FeJ mice [5], as well as in mouse strains with genetic defects in macrophage activation, C3H/HeJ [5] and P/J [6]. For example, C3H/HeJ mice do not acquire tumoricidal macrophages in response to vaccination with live bacillus Calmette-Guérin (BCG) [7], but we have shown that attenuated salmonella induces such activation [5]. Further, macrophages taken from salmonella-vaccinated mice are also microbicidal, as shown by their capacity to retard growth of *Leishmania major in vitro* [5]. Mice

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immunized with SL3235 are resistant to challenge with *Listeria monocytogenes* from days 3 to 21 post-vaccination, which provides indirect evidence for induction of activated macrophages [8]. Additional direct evidence of activation is that macrophage-rich fractions of immune mouse spleens transfer anti-salmonella resistance [8]. The macrophage-activating properties of live SL3235 cannot be attributed solely to their endotoxin content, as acetone-killed cells (AKC) of salmonella are not effective in inducing tumoricidal macrophages in C3H/HeJ mice, but can prime them to tumoricidal activity after exposure to treatment with interferon-gamma *in vitro* [5].

Biological response modifiers (BRMs) have had beneficial effects in treatment of some cancers. A mixed bacterial vaccine containing gram-negative *Serratia marcescens* and *Streptococcus pyogenes* was one of the first BRMs used in the treatment of cancer and has been shown in a recent clinical study to enhance the immune responses of terminally ill patients [9,10]. BCG has been extensively studied as a BRM and macrophage-activating agent, and has been used as an immunotherapeutic agent in treating cancer [11-17]. In the studies reported in this paper, we have used the attenuated strain of *S. typhimurium* in a manner similar to that of BCG, to test its capacity to retard tumor growth *in vivo*, since the salmonella were shown to be more potent than BCG in inducing tumoricidal macrophages in C3H/HeJ mice. The neoplastic system chosen, the subcutaneous growth of a murine plasmacytoma, TEPC-183, was one that has been extensively studied [1-3]. When injected subcutaneously into syngeneic BALB/c mice, it rarely invades surrounding tissue, even when tumors reach up to 10–12 cm^2 at the base, and there is no firm evidence of metastases. It kills mice by its massive size at the injection site. It never regresses spontaneously [1-3,18]. Our data show that SL3235 is able to retard growth of the tumor and to increase the survival of tumor-bearing mice when given therapeutically and at a site distant from the tumor. Non-viable salmonella were not effective as an immunotherapeutic agent.

MATERIALS AND METHODS

Mice

Eight- to 10-week-old BALB/cAnlcR (BALB/c) mice of both sexes were obtained from the Institute for Cancer Research (Philadelphia, Pa.). They were fed *ad libitum* with Purina mouse chow.

Plasmacytoma

TEPC-183, a murine IgM-producing plasmacytoma, was kindly supplied by Dr Zolla-Pazner [19] (NYU Medical College). Tumors were maintained by serial transfer [1]. After removal, the solid tumor (TEPC-183 (IgMκ)) was placed in chilled Hanks' solution, cut into small pieces, and gently pressed through a plastic screen onto a Petri dish containing Hanks' solution, pH 7.4 (Grand Island Biological Laboratories, Grand Island, NY). The suspension was then filtered through nylon mesh, washed once, and resuspended in chilled Hanks' solution. An aliquot of the tumor cell suspension was diluted 1:10 in 0.1% Trypan Blue, counted in a hemocytometer, and the volume adjusted to contain 10^5 viable cells in 0.5 ml for intraperitoneal (i.p.) or subcutaneous (s.c.) injection. The s.c. injection was administered intrascapularly. Tumor size was measured by a series of calibrated rings.

Salmonella

S. typhimurium, strain SL3235, is an *aroA*$^-$ mutant derived by transduction using a Tn10 transposon by Hoiseth and Stocker [20] and kindly supplied by Dr Stocker. The *aroA*$^-$ lesion rendered the strain avirulent and nonreverting. It has been used extensively in our laboratory for studies on salmonella immunity [8,21-24].

For injection of live salmonella, lyophils were rehydrated with brain-heart infusion broth and plated on trypticase soy agar. Brain heart infusion broth (10 ml) was inoculated with five colonies from the blood agar plate and incubated for 4 h at 37°C. A 5-ml sample of this log-phase culture was then transferred to 50 ml of brain heart infusion broth and incubated for an additional 2 h at 37°C with shaking. The culture was placed on ice, the number of organisms was estimated by counting in a Petroff-Hauser chamber, and dilutions were made in saline to achieve the desired concentration. The exact number of *S. typhimurium* injected was determined by duplicate plate counts. Salmonella were injected intraperitoneally or intralesionally in a volume of 0.5 ml.

Acetone-killed and dried SL3235 were prepared by standard procedures [25].

Statistical analysis

Differences in tumor sizes among treated and experimental groups were assessed by Student's *t*-test. Differences in survival among groups were assessed by Fisher's Exact test for 2 x 2 tables [26]. Differences in mean time to death (MTD) were assessed by the Mann and Whitney modification of the Wilcoxon rank order test [27]. Values for all tests were taken as significant at $p \leq 0.05$.

RESULTS

Table 1 shows that 2×10^5 SL3235 given intraperitoneally 30 min post-subcutaneous inoculation of

Table 1. Effect of intraperitoneal attenuated *S. typhimurium* SL3235 on subcutaneous growth of TEPC-183

Treatment	Tumor size (cm^2)		MTD (range)
	Day 12	Day 14	
Saline[a]	3.67 ± 1.5	7.8 ± 1.5	18.2 (17–19)
2×10^5 SL3235[a]	0.83 ± 0.26[b]	3.17 ± 0.86[b]	25.5[c] (17–34)

[a] Six mice per group were injected with 1.5×10^6 TEPC-183 cells subcutaneously in the intrascapular region. Thirty minutes post-tumor injection one group received 2×10^5 SL3235 intraperitoneally and the other group received sterile saline.
[b] $p \leq 0.001$ versus saline.
[c] $p \leq 0.05$ versus saline.
MTD = mean time to death.

TEPC-183 retarded tumor growth (0.83 cm^2 in treated versus 3.67 cm^2 in controls at day 12, $p \leq 0.001$) and prolonged mean time to death (25.5 days *versus* 18.2 days, $p \leq 0.05$). To simulate more closely a clinical situation in which the therapy would be administered after the tumor reached a palpable mass, the effect of delaying the time of injection of SL3235 was evaluated, and the vaccine was given intralesionally (Table 2). The results presented show that when SL3235 was given as late as the sixth day post-tumor inoculation, the attenuated salmonella were able to retard tumor growth significantly and prolong mean time to death ($p \leq 0.01$). When therapy was administered on the eighth day post-tumor, it was less effective, although it still had a significant effect on

Table 2. Effect of therapeutic injection of *S. typhimurium* SL3235 given intralesionally

Treatment[a]	Day post-tumor inoculation		Tumor size[b] (cm^2)	MTD (range)	Survivors/total[c]	
	Day 6	Day 8			Day 27	Day 33
Group I	–	Saline	5.0 ± 1.26	17.4 (15–20)	0/8	0/8
Group II	SL3235	–	2.6 ± 1.28[d]	30.2[d] (15–48)	7/8[e]	1/8
Group III	–	SL3235	3.75 ± 0.46[f]	22.4[f] (17–27)	1/8	1/8

[a] Twenty-four mice were injected with 1.5×10^6 TEPC-183 subcutaneously and divided into three groups of eight mice each. Groups II and III received 2×10^5 SL3235 intralesionally on days 6 or 8, respectively, and Group I received saline.
[b] Measurements taken on day 16 post-tumor inoculation.
[c] On day 27 post-tumor inoculation.
[d] $p \leq 0.01$ versus saline.
[e] $p \leq 0.001$ versus saline.
[f] $p \leq 0.05$ versus saline.
MTD = mean time to death.

Table 3. Comparison of efficacy of intralesional versus intraperitoneal injection of 1.5×10^6 SL3235

Groups	Treatment[a]	MTD (range)	Survivors/total		Tumor size (cm^2, day 22)
			Day 22	Day 36	
I	SL3235, i.l.	26.4 (19–34)	4/8	0/8	5.8 ± 0.84
II	Saline, i.l.	24.3 (18–39)	3/8	1/8	7.0 ± 1.41
III	SL3235, i.p.	36.6 (15–49)	11/13	5/13	4.2 ± 1.53[b]
IV	Saline, i.p.	23.2 (18–46)	5/7	2/7	6.8 ± 2.95

[a] Mice were injected with 1.5×10^6 TEPC-183 cells as described. On day 7 post-tumor inoculation, injections were either intralesional (i.l.) or intraperitoneal (i.p.) with saline (Groups II and IV) or with 1.5×10^6 SL3235 (Groups I and III).
[b] $p \leq 0.05$
MTD = mean time to death.

Table 4. Comparison of different doses of live and acetone-killed salmonella

Group[a]	Treatment	Dose	MTD (range)	% Survival day 33	Tumor size (cm^2, day 17)
I	Live SL3235	2×10^6	29.8[b] (24–46)	33%	3.50 ± 0.84[b]
II	Live SL3235	2×10^5	36.6[b] (31–46)	50%	3.03 ± 1.63[b]
III	Live SL3235	2×10^4	36.4[b] (24–46)	67%[c]	5.67 ± 1.03[b]
IV	AKC SL3235	100 µg	21.5 (18–26)	0%	6.17 ± 1.17
V	AKC SL3235	50 µg	23.4 (18–34)	33%	6.20 ± 0.95[b]
VI	AKC SL3235	25 µg	22.8 (17–31)	0%	7.00 ± 1.41
VII	Saline	–	20.3 (18–24)	0%	7.00 ± 0

[a] Six mice per group were injected with salmonella 3 days post-tumor inoculation.
[b] $p \leq 0.01$ versus saline.
[c] $p \leq 0.05$ versus saline.
AKC = acetone-killed salmonella.
MTD = mean time to death.

tumor burden ($p \leq 0.05$). At 27 days after tumor inoculation, only one mouse had succumbed in the group which had received salmonella on the sixth day, but all untreated mice and all but one of the mice receiving salmonella on the eighth day, had died.

A comparison of the efficacy of intralesional *versus* intraperitoneal therapy was carried out in mice treated 7 days post-tumor injection, using a 10-fold higher dose of SL3235 (1.5×10^6 cells). As shown in Table 3, the higher dose of salmonella was not effective, as measured by survival or MTD, when given intralesionally or intraperitoneally at 7 days post-inoculation, with the exception of the tumor size on day 22 in the i.p. group. In view of the lesser efficacy of the higher dose of the salmonella, as shown in Table 3 as compared with Tables 1 and 2, a dose response study was carried out (Table 4). In addition, the efficacy of live *versus* acetone-killed salmonella was also tested. Salmonella were injected intraperitoneally on day 3 post-subcutaneous tumor inoculation. As shown in Table 4 and Figure 1, nonviable, acetone-killed (AKC) salmonella were ineffective in increasing survival or in prolonging mean time to death. In contrast, live salmonella, at all three doses, increased mean time to death. The highest dose, 2×10^6 cells, was again not protective in terms of percent survival when scored at day 33 post-tumor inoculation. The lowest dose of salmonella (2×10^4 organisms) enhanced survival ($p \leq 0.05$). The intermediate dose (2×10^5) was less effective ($p \leq 0.09$). All three doses significantly retarded tumor growth.

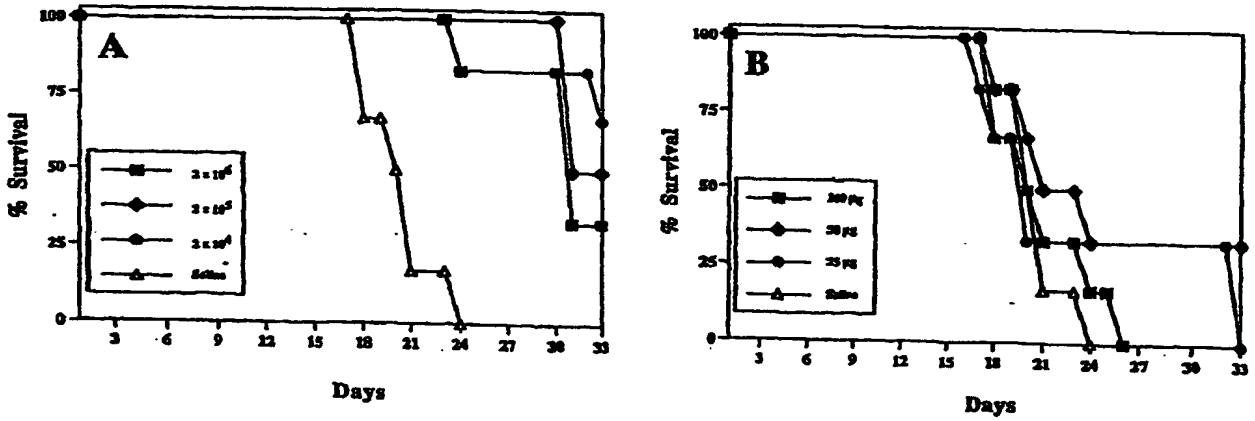

Fig. 1. Survival of mice injected intraperitoneally 3 days post-subcutaneous tumor inoculation with varying doses of live (panel A) or acetone-killed (AKC) (panel B) salmonella.

DISCUSSION

These studies show that a live, attenuated salmonella vaccine, but not acetone-killed cells, can act as an immunotherapeutic agent to retard growth of a murine plasmacytoma, leading to lower tumor burden, increased mean time to death, and increased survival. Three aspects of these results are particularly significant. First, the salmonella vaccine was effective against a rapidly growing, immunosuppressive tumor, TEPC-183, which does not spontaneously regress [1-3]. Second, the attenuated salmonella retarded tumor growth when given at a distant site (intraperitoneally) from the tumor mass, as well as intralesionally. The effect is analogous to that of a mixed bacterial vaccine which was administered to humans intramuscularly and subcutaneously distant from the tumor site [9,10]. Third, the salmonella vaccine was effective not only against tumor growth, but also against well established tumors which have reached 20–30 cm^2 at the base. Thus, an increased survival time was observed when the delay in salmonella injection was 3 days (Table 4) or 6 days (Table 2) post-tumor. The ability of the vaccine to have a therapeutic effect is compatible with potential for clinical use.

The present studies do not address the mechanism of the therapeutic effect of salmonella. However, other work from our laboratory has shown that live SL3235 induces tumoricidal macrophages that are cytotoxic for B16 melanoma cells, P815 mastocytoma cells, and a TU-5 fibrosarcoma line [5]. Furthermore, the live salmonella were able to induce cytotoxic activity in peritoneal macrophages of C3H/HeJ mice, whereas BCG alone is not effective in this mouse strain [5]. In this regard, we had previously observed that nonviable SL3235 could only prime C3H/HeJ macrophages for tumoricidal activity, but were not sufficient to induce tumoricidal activity [5]. Interestingly, in the present epxeriments, the need for living salmonella was demonstrated. Thus, lipopolysaccharide alone is not sufficient to induce tumor cytotoxicity.

Activated macrophages can damage tumor targets by secretion of tumor necrosis factor (TNF) [28] or by production of nitric oxide (NO) [29]. Live salmonella are potent inducers of NO in splenic macrophages [24] and in peritoneal macrophages [30] as is BCG [31]. We have shown that NO mediates toxicity against a Renca renal adenocarcinoma line [30] and also against P815 mastocytoma cells (unpublished results). Thus, one mechanism by which the salmonella might retard TEPC-183 plasmacytoma growth is via induction of NO-producing macrophages. Kolb *et al.* [32] had postulated that macrophages might mediate the immunosuppression observed in TEPC-bearing mice, although studies by Patel and Havas did not support that conclusion [33]. It is interesting that the lower doses of salmonella (2×10^4, 2×10^5) seemed to be more effective in prolonging survival in TEPC-bearing mice than the highest dose (2×10^6), an observation which would be consistant with reports in the literature that an excess of tumor-associated macrophages can also be deleterious [34]. The observation that mice receiving 2×10^4 live SL3235 had longer survival but larger tumor burdens (Table 4) is not easily explained. In a study of cellular changes in TEPC-183-bearing mice 14 days after tumor inoculation, it was found that cells expressing MAC-1 antigen and nonspecific esterase activity increased six-fold in the spleen with increasing tumor growth [35]. We have also shown that activated macrophages can be immunosuppressive via production of excessive NO [21-24,36], and have postulated that there may be a narrow dose window between beneficial and deleterious effects of activated macrophages induced by salmonella and other BRMs [37-39].

CONCLUSIONS

Our studies show that attenuated salmonella have potential as an immunotherapeutic agent. Because they can supply an LPS signal in addition to other signals provided by living intracellular bacteria, salmonella may be superior on theoretical grounds to BCG as an immunotherapeutic agent. There are few anti-cancer drugs that totally destroy established tumors, so that even a modest reduction in rate of increase of tumor size and an increase in survival time are significant. With the increasing focus on BRMs, the use of salmonella should be explored further.

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Tumor-targeted *Salmonella* as a Novel Anticancer Vector[1]

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ABSTRACT

There has been little investigation of bacteria as gene delivery vectors. Here, we demonstrate that genetically engineered *Salmonella* have many of the desirable properties of a delivery vector, including targeting of multiple tumors from a distant inoculation site, selective replication within tumors, tumor retardation, and the ability to express effector genes, such as the herpes simplex virus thymidine kinase (*HSV TK*). When wild-type *Salmonella* were introduced into melanoma-bearing mice, the bacteria were found within the tumor at levels exceeding 10^9 per g, although as pathogens, they caused the death of the mice. However, when attenuated, hyperinvasive auxotrophic mutants were used, the tumor-targeting and amplification phenomena were retained, whereas their pathogenicity was limited. With such attenuated strains, the tumor:liver ratios ranged between 250:1 and 9000:1. When these auxotrophs were inoculated i.p. into C57B6 mice bearing B16F10 melanomas, they suppressed tumor growth and prolonged average survival to as much as twice that of untreated mice. A plasmid containing the *HSV TK* gene with a β-lactamase secretion signal was constructed that, when expressed, resulted in translocation to the periplasm and phosphorylation of the prodrug ganciclovir. Melanoma-bearing animals inoculated with HSV TK-expressing *Salmonella* showed ganciclovir-mediated, dose-dependent suppression of tumor growth and prolonged survival in addition to that seen with bacteria alone. The results demonstrate that attenuated *Salmonella* would be useful both for inherent antitumor activity and delivery of therapeutic proteins to cancer cells *in vivo*.

INTRODUCTION

Salmonella offer several potential advantages as anticancer vectors. They can grow under either aerobic or anaerobic conditions such as those that occur within solid tumors (1–5); they express specialized systems for invasion into and survival within both epithelial cells and macrophages (6–11); and there is a vast body of knowledge and powerful genetics in the *Enterobacteriaceae*. Thus, we investigated the invasive capacities of *Salmonella* toward melanomas and cells of other solid tumors, both in culture and following implantation in mice. Finding that a highly virulent wild-type *Salmonella* invaded these cells *in vitro* and targeted and amplified within tumors *in vivo*, we sought to develop this strain as an antitumor vector.

We first isolated derivatives that, unlike wild type, were not repressed in invasion following aerobic growth and, therefore, remained invasive toward cancer cells under either aerobic or anaerobic growth conditions (2–5). However, for a pathogen such as *Salmonella* to be useful as an anticancer vector, it must also be attenuated in virulence so that potential harm to the host is minimalized. Previous work demonstrated that attenuation of *Salmonella* can be achieved through auxotrophic mutations, such as those affecting the biosynthesis of purines (12) or aromatic amino acids (13), or combinations of such mutations (14). We reasoned that the environment of the tumor might provide essential nutrients to the auxotrophs, for example, in necrotic spaces or within actively dividing cells of the tumor. Thus, auxotrophy would not only reduce their virulence but could potentially provide a mechanism for their selective population and amplification within solid tumors.

Below, we detail the development of aerobically invasive *Salmonella* auxotrophs that are attenuated in mice but show pronounced tumor targeting and amplification capabilities. Additionally, we show that the strains can be genetically engineered to express effector genes, such as that encoding HSV TK,[4] which converts of the inactive prodrug GCV into its activated phosphorylated form, resulting in antitumor activity *in vivo*.

MATERIALS AND METHODS

Cell Lines and *in Vitro* Invasion Assays

The bacteria were wild-type *Salmonella typhimurium* strain 14028 (ATCC CDC6516-60) and the following auxotrophic derivatives: YS72 (*pur*$^-$); YS721 (*pur*$^-$, *ilv*$^-$); YS7211 (*pur*$^-$, *ilv*$^-$, *arg*$^-$); YS7212 (*pur*$^-$, *ilv*$^-$, *ura*$^-$); and YS7213 (*pur*$^-$, *ilv*$^-$, *aro*$^-$). Human melanoma line M2 was provided by Dr. C. Cunningham (Harvard Medical School, Cambridge, MA). B16F10 cells were provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX). All other cell lines were obtained from the American Type Culture Collection. Bacterial invasion of cultured animal cells was carried out in Corning tissue culture flasks (25 cm^2) with the use of gentamicin sulfate, as modified from Lee and Falkow (4). Following 15- or 30-min invasion periods and 30-min incubations with 50 μg/ml gentamicin, animal cells were quantitated in a Coulter counter (Coulter Electronics, Inc.), and bacteria were quantitated by serial dilutions on LB agar. Data were expressed as the number of infecting (gentamicin-resistant) bacteria/10^6 animal cells.

Isolation of Auxotrophs and Testing of Virulence in Mice

Strains 14028 and YS72 were mutagenized with 50 μg/ml nitrosoguanidine (20 min at 37°C) and UV irradiation (50 J/m^2, λ = 254 nm; Ref. 15). In one study, three separate *pur*$^-$ auxotrophic mutant clones were isolated from 14028, and three separate *pur*$^+$ revertant clones were isolated from YS72 and tested for virulence in mice and for invasion potential toward M2 melanoma cells *in vitro*. In another study using YS72 as the parental strain, polyauxotrophs YS721, YS7211, YS7212, and YS7213 were constructed and also tested for virulence and invasiveness. Virulence was tested by injecting groups of C57B6 mice i.p. with 10^6 bacteria and measuring the mean time to death (12). This method was chosen over that of the LD_{50} method because it yielded reproducible, statistically significant results with fewer mice. C57BL6 mice (n = 8–12 animals) were injected i.p. with each isolate, the mice were allowed to eat and drink *ad libitum*, and the cages were monitored for dead or moribund mice. All surviving animals were euthanized 10, 30, or 60 days after inoculation with bacteria, depending on the experiment. Data were expressed as mean ± SD survival after inoculation with bacteria (in days).

Implantation of Tumors and Quantitation of Bacteria

C57B6 and DBA/2J mice were inoculated s.c. in the left shoulder region with 5×10^5 B16F10 and Cloudman S91 mouse melanoma cells, respectively. BALBc *nu/nu* mice were inoculated with 2×10^7 cells of human lung carcinoma A549, human colon carcinoma HCT 116, human breast carcinoma

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[4] The abbreviations used are: HSV TK, herpes simplex virus thymidine kinase; GCV, ganciclovir; LB, Luria broth; cfu, colony-forming unit(s); EM, electron microscopy; TK, thymidine kinase; T/C, treated *versus* control; LPS, lipopolysaccharide; i.t., intratumoral.

BT20, human renal carcinoma CRL 1611, or human hepatoma HTB 52. When palpable tumors developed, mice were further inoculated i.p. with *Salmonella* isolate YS72 at the cfu indicated. At designated times, the animals were sacrificed, and the tumors and livers were removed and weighed. A central portion of the tumor was prepared for microscopy, and the remainder of the tumor was homogenized in 5 volumes of LB per g of wet weight tissue and quantitated for bacteria by serial dilution on LB agar plates.

Bacterial Effects on Tumor Growth and Survival

Inoculi of $1-4 \times 10^6$ cfu/mouse i.p. of the polyauxotrophs were not lethal for at least 28 days, and some strains of mice tolerated inoculi as high as 5×10^7 cfu. Doses in the range of 10^6 cfu were chosen as the most useful for investigating the several different factors that needed to be taken into account: virulence, tumor targeting, amplification, and growth suppression. Eight days following tumor implantation, mice were further inoculated i.p. with 4×10^6 cfu of YS721, YS7211, YS7212, or YS7213. Ten days following inoculation of bacteria, mice were routinely given Baytril (enrofloxacin, 0.2 mg/ml in drinking water) for a total of 2 weeks, a treatment that, in comparative studies not shown, resulted in a moderate increase in survival of mice. Tumor growth was assessed by periodic caliper measurements (in mm). Tumor volume was computed by the formula: length × width × height × 0.5236 = volume (in mm^3). Animals were euthanized when their tumors reached 4000 mm^3 or when they became moribund.

Microscopic Analyses

For light microscopic analyses, portions of the tumor were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E or tissue Gram's stain (Brown-Brenn stain). For EM, the tissue was fixed in half-strength Karnovsky's fixative for 6 h at 4°C, followed by washing in cacodylate buffer overnight, postfixed with 1% OsO_4 and 1.5% potassium ferrocyanide in cacodylate buffer for 2 h, and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and photographed through a Zeiss 109 EM.

Cloning and Expression of HSV TK in *Salmonella typhimurium*

PCR. Plasmid DNA of the vector pHETK2 (16) was prepared by alkaline lysis, phenol-chloroform extraction, and ethanol precipitation. PCR primers based on the complete sequence for *HSV TK* (17) were: forward, 5'-GATCATGCATGGCTTCGTACCCCGGCC-3'; and reverse, 5'-CTAGATGCATCAGTGGCTATGGCAGGGC-3', corresponding to bases 310–328 (forward) and 1684–1701 (reverse) of the published sequence, with added GATCATGCAT or CTAGATGCAT sequences (*NsiI* site and spacer) at the 5' end of each primer. Each 25-μl reaction mixture contained 50 ng of DNA template, 10 pmol of each primer, 100 mM deoxynucleotide triphosphates, 1.5 mM Mg, and 0.5 units of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT). Amplification was performed by 35 cycles of 94°C for 1 min; 50°C for 15 s; 55°C for 1 min; and 72°C for 2 min. The band of the correct size was cloned into (*a*) pBluescript II KS^+ and sequenced with T3 and T7 primers to confirm the correct DNA had been cloned and (*b*) p279 cut with *PstI*, which provides the β-lactamase signal sequence (18). Transformants were screened using a probe generated from the original template by random priming (Boehringer Mannheim, Indianapolis, IN) using [α-^{32}P]dCTP. Positive clones were further screened by immunoblot.

SDS-PAGE and Immunoblot. SDS-PAGE was performed on bacterial lysates according to Weber and Osborn (19). Immunoblots were performed according to Towbin *et al.* (20). Primary anti-TK antibodies (a gift of William Summers, Yale University, New Haven, CT) were generally used at a 1:1000 dilution. Secondary antimouse antibodies were alkaline phosphatase conjugates (Promega, Madison, WI) used at a 1:7500 dilution, and antibody incubations were followed by nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate colorimetric detection (Promega).

TK Assay. TK activity was assayed using a modification[5] of the method of Summers and Summers (21) containing 0.2 mM I-dC, 0.01 mM ^{125}I-dC, 10 mM ATP, 0.6 mg/ml BSA, 10 mM $MgCl_2$, 25 mM NaF, and 100 mM sodium phosphate buffer, pH 6.0. Five ml of the enzyme extract were combined with 20 ml of the reaction mix and incubated at 37°C for 1 h, bound to DE81 paper (Whatman), and washed, and the associated radioactivity was determined in a gamma counter.

***Salmonella* Transformation.** Transformation of *Salmonella* strains was performed by electroporation as described (22). Plasmids transfected into *Salmonella* included pHETK2 (16), p279 (18), and two independent isolates of β-lactamase fusions, p5-3 and p21A-2. *Salmonella typhimurium* strains that were transfected were 14028, YS72, YS7211, YS7212, and YS7213.

Fractionation of Bacteria and Isolation of Periplasmic Fractions

Periplasmic fractions were collected using a modified osmotic shock procedure (23, 24). Cultures were grown overnight at 37°C with shaking in medium consisting of LB with either 100 μg/ml ampicillin or 10 μg/ml tetracycline. One ml of bacterial culture was pelleted and resuspended in 100 μl of Tris-acetate (0.1 M). Eight ml of lysozyme (2 mg/ml) were added, the mixture was kept at room temperature for 10 min, 100 μl of ice-cold H_2O containing 1 mM phenylmethylsulfonyl fluoride was added, and the mixture was further incubated on ice for 5 min. Four μl of $MgSO_4$ (1 M) were added to stabilize the spheroplasts, which were then pelleted in a microfuge for 40 s at 12,000 × *g*. The spheroplast pellet and the supernatant periplasmic fraction were compared with the whole unfractionated bacteria by gel electrophoresis and immunoblotting.

Treatment with GCV

Eight days post-tumor implantation, some mice were further inoculated i.p. with 10^6 cfu of strain YS7211 bearing *HSV TK* gene-containing plasmid p5-3 with a β-lactamase secretory signal sequence. At 11 days post-tumor implantation, GCV (GCV sodium, Cytovene; Syntex Laboratories, Palo Alto, CA) was inoculated i.p. into groups of mice under the following protocols: (*a*) 330 mg/kg (2.5 mg, day 11; 1.25 mg, day 12; 2.5 mg, day 18; and 1.25 mg, day 19); (*b*) 220 mg/kg (2.5 mg, day 11; 2.5 mg, day 12); (*c*) 165 mg/kg (2.5 mg, day 11; 1.25 mg, day 12); (*d*) 110 mg/kg (1.25 mg, day 11; 1.25 mg, day 12); and (*e*) 55 mg/kg (1.25 mg, day 11). At 18 days post-tumor implantation (10 days post-bacterial inoculation), all animals were given enrofloxacin (Baytril, 0.2 mg/ml) in their drinking water, and they were maintained on this antibiotic for 2 weeks.

Genetic Stability of Strains

To assess the stability of the auxotrophic phenotypes bacteria from tumor, homogenates were replicate-plated onto minimal medium agar supplemented with the appropriate nutritional additives to monitor for the presence of the auxotrophic mutations or with tetracycline on LB plates for retention of the Tet^{res} plasmid. For each strain, YS7211, YS7212, and YS7213, 50 of 50 bacterial clones recovered from the tumor homogenates displayed the expected auxotrophic phenotypes 2 days postinoculation. In tests for plasmid retention, 10 days after inoculation of YS7211 with plasmid p5-3, 27 of 33 clones recovered from tumors were Tet^{res}, indicating >80% retention of the HSV TK plasmid *in vivo*. Further, for each of the three auxotrophs, three of three Tet^{res} clones isolated from such tumor homogenates expressed TK activity, indicating that, in addition to Tet^{res}, the TK gene was also retained on the plasmid for at least 10 days in mice (not shown).

RESULTS

Isolation of Aerobic Hyperinvasion Mutants. Invasion of *Salmonella* into animal cells is regulated by a number of environmental factors, including oxygen, osmolarity, and pH (2–5). For example, mutants of the *hil* locus have been described previously that were hyperinvasive under normally repressive aerobic conditions (2, 4–5). Using similar techniques as those used for *hil* mutants, we sought to isolate mutants showing enhanced invasive capacities toward human melanoma cells when the bacteria were growing aerobically in early logarithmic phase. ATCC 14028 was chosen as the wild type because it was known to be highly virulent in mice and we found it to be inherently invasive toward human melanoma cells in culture. Strain 14028 was mutagenized so that the frequency of auxotrophic mutants

[5] P. Tung and W. C. Summers, personal communication.

reached 2% (15). The mutagenized population was grown to early logarithmic phase under aerobic conditions and subjected to four cycles of invasion into and recovery from melanoma cells. Four of 150 isolates tested were found to be hyperinvasive compared to wild type, and the most striking of these, YS72, was chosen for further study. The invasive characteristics of YS72 showed a phenotype similar to that of *hil* mutants in that invasion of wild-type cells was repressed under aerobic conditions and induced under anaerobic conditions, whereas invasion of YS72 was induced following growth under either condition (Table 1). Although YS72 was isolated by cycling through melanoma cells, it was also hyperinvasive compared to wild type toward human carcinomas of the lung, prostate, kidney, liver, and breast, in *in vitro* assays following aerobic growth (data not shown).

Analyses of growth in minimal medium supplemented with various nutrients revealed that YS72 was *pur*$^-$, requiring adenine and vitamin B1, although no selective pressure had been applied for isolation of the Pur$^-$ phenotype. This phenotype is known to reduce virulence of *Salmonella* in mice (12–14), and upon testing, YS72 was indeed found to be less virulent than wild type (Table 2). That is, whereas non-tumor-bearing C57B6 mice inoculated i.p. with 10^6 cfu of wild type survived only 3 ± 0.5 days (mean ± SE), those inoculated with YS72 survived 5.8 ± 1 days, suggesting that the Pur$^-$ phenotype accounted for the reduced virulence of YS72. In confirmation, a series of *pur*$^-$ derivatives of 14028 had decreased virulence, similar to YS72, whereas *pur*$^+$ derivatives of YS72 had increased virulence, similar to 14028 (Table 2). Through *in vitro* invasion assays, it was found that the aerobic hyperinvasive phenotype was expressed in both the YS72 *pur*$^-$ and the *pur*$^+$ derivatives and was, therefore, unrelated to the *pur*$^-$ phenotype of YS72 (data not shown).

Attenuation and Selective Amplification in Tumors. YS72 was subjected to successive rounds of mutagenesis, and the following auxotrophs were constructed. An *ilv*$^-$ (isoleucine/valine) derivative of YS72, designated YS721, was isolated, and three additional auxotrophs were obtained from YS721: one additionally requiring arginine, YS7211; a second additionally requiring uracil, YS7212; and a third additionally requiring precursors for aromatic amino acid synthesis, YS7213. Virulence was tested by injecting groups of mice i.p. with 10^6 bacteria and measuring the mean time to death (Ref. 12; see "Materials and Methods"). The polyauxotrophs were all less virulent than YS72, in that about 50% (8/17) of non-tumor-bearing C57B6 mice inoculated with YS721 and about 90% (33/36) inoculated with YS7211, YS7212, or YS7213 survived past 30 days with no antibiotic treatment (Table 2). Further, polyauxotrophs YS721, YS7211, and YS7212 retained the hyperinvasive phenotype. However, YS7213 was markedly less invasive *in vitro* than any of the other strains, being about 30-fold less so than the wild type, following either anaerobic or aerobic growth (data not shown).

The wild-type and auxotrophic strains were examined for tumor and liver distribution after i.p. inoculation into C57B6 mice bearing B16F10 melanomas (Table 3). Prior tests of 10^1-10^8 cfu/mouse indicated that 10^6 cfu was most useful for observing tumor and liver infectivity for at least 2 days, while, at the same time, maintaining the viability of mice injected with virulent strains such as 14028 and YS72 (data not shown). By 5 h, bacteria were found at low levels in both liver and tumor, with tumor:liver ratios of 1:2 for wild type and 2:1 for YS72. Remarkably, by 2 days, each of the strains tested had selectively amplified within tumors, such that the number within the tumors for each strain was far more than that of the original inoculum of 10^6 cfu, and tumor:liver ratios were as high as 9000:1. However, the most striking finding in these studies occurred at 4 days. Although wild type and YS72 were lethal to the mice at 4 days, polyauxotrophs YS721, YS7211, YS7212, and YS7213 were not, and each displayed densities of greater than 10^9 cfu/g tumor, with tumor:liver ratios between 250:1 and 6000:1. These results indicated that such polyauxotrophs, capable of tumor amplification yet expressing reduced virulence, should be useful as safe tumor vectors in mice.

Tumor Retardation and Prolonged Survival. Additional studies established that YS721, YS7211, YS7212, and YS7213 each retarded tumor growth and, except for YS721, which was only partially attenuated, they prolonged survival of tumor-bearing mice (Fig. 1). For the experiment in Fig. 1, the average time for tumors to reach 1 g was 18 days for control animals, 31 days for animals treated either with YS7213 and YS7211, and 45 days for YS7212. Retardation of tumor growth paralleled survival times (Table 4). The order of efficacy for

Table 1 *Invasion of wild-type Salmonella strain 14028 and hyperinvasive mutant YS72 following growth under aerobic and anerobic conditions*

Bacteria were grown aerobically in LB or anaerobically in N_2-bubbled broth for at least three doublings to A600 = 0.5, diluted to 10^6 cfu in DMEM/10% fetal bovine serum, and allowed to invade M2 melanoma cells for 15 min under aerobic conditions. The invasive characteristics of YS72 showed a phenotype similar to that of *hil* mutants (2, 4–5). Each point represents the mean ± SD for triplicate determinations. The experiments were repeated several times with similar results.

	Salmonella/10^6 human cells		
Growth condition	14028	YS72	YS72:14028
Aerobic	$3.4 \pm 1.7 \times 10^2$	$4.5 \pm 0.3 \times 10^4$	130:1
Anaerobic	$2.7 \pm 0.6 \times 10^4$	$4.7 \pm 0.2 \times 10^4$	1.7:1

Table 2 *Survival of C57B6 mice injected with auxotrophic mutants of Salmonella typhimurium*

Points represent the mean ± SD for n = 8–12 animals. See "Materials and Methods" for further description of strains.

Strain	Phenotype	Mean survival (days ± SD)	No. of survivors >10 days	No. of survivors >30 days
14028	Wild type	3.0 ± 0.5	NA[a]	NA
YS72	Hyperinvasive, pur$^-$	5.8 ± 1.0	NA	NA
YS72-P1	Pur$^+$	3.9 ± 0.4	NA	NA
YS72-P2	Pur$^+$	3.9 ± 1.3	NA	NA
YS72-P3	Pur$^+$	4.1 ± 0.9	NA	NA
14028-P1	Pur$^-$	6.8 ± 1.5	NA	NA
14028-P2	Pur$^-$	NA	4/8	ND
14028-P3	Pur$^-$	NA	5/8	ND
YS721	Pur$^-$, Ilv$^-$	NA	10/11	6/11
YS7211	Pur$^-$, Ilv$^-$, Arg$^-$	NA	8/8	7/8
YS7212	Pur$^-$, Ilv$^-$, Ura$^-$	NA	8/8	6/8
YS7213	Pur$^-$, Ilv$^-$, Arg$^-$	NA	8/8	8/8

[a] NA, not applicable; ND, not done.

Table 3 *Tumor and liver distribution of Salmonella at 5 h and 2 and 4 days after i.p. inoculation of 10^6 cfu*

Points are for C57B6 mice bearing B16F10 melanoma tumors, representing mean ± SD for n = 3 (5 h) and n = 5 (2 and 4 days) animals.

	Salmonella/g tissue		
Time postinoculation	Tumor	Liver	Tumor:Liver
5 h			
14028WT	$7.1 \pm 2.2 \times 10^4$	$1.3 \pm 7.6 \times 10^5$	1:2
YS72	$1.2 \pm 1.3 \times 10^5$	$5.5 \pm 4.8 \times 10^4$	2:1
2 days			
14028WT	$6.5 \pm 6.8 \times 10^9$	$2.4 \pm 2.8 \times 10^7$	270:1
YS72	$1.7 \pm 1.2 \times 10^9$	$1.9 \pm 2.3 \times 10^5$	9000:1
YS721	$8.7 \pm 3.1 \times 10^8$	$4.2 \pm 3.6 \times 10^6$	210:1
YS7211	$3.3 \pm 3.0 \times 10^7$	$8.1 \pm 8.4 \times 10^5$	41:1
YS7212	$3.9 \pm 7.3 \times 10^7$	$1.1 \pm 0.8 \times 10^6$	35:1
YS7213	$1.5 \pm 2.8 \times 10^8$	$4.0 \pm 3.1 \times 10^5$	375:1
4 days			
14028WT	Moribund/dead		
YS72	Moribund/dead		
YS721	$3.2 \pm 1.5 \times 10^9$	$4.7 \pm 6.9 \times 10^6$	680:1
YS7211	$1.6 \pm 2.2 \times 10^9$	$6.3 \pm 9.9 \times 10^6$	253:1
YS7212	$1.1 \pm 7.4 \times 10^9$	$5.1 \pm 8.6 \times 10^5$	2200:1
YS7213	$1.3 \pm 2.5 \times 10^9$	$2.2 \pm 6.9 \times 10^5$	5900:1

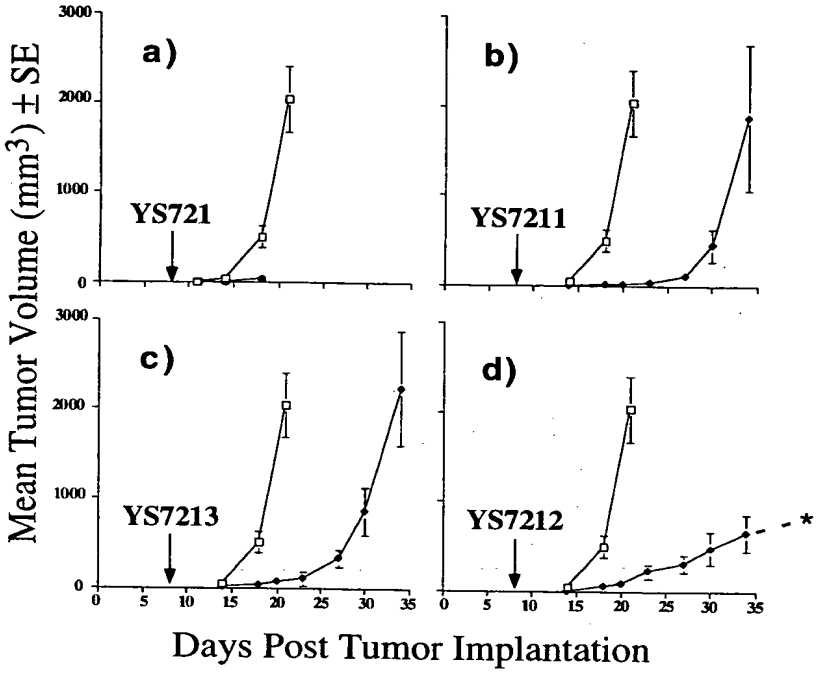

Fig. 1. Growth of B16F10 melanomas in C57B6 mice with (◆) and without (□) inoculation of strains YS721 (*a*); YS7213 (*b*); YS7211 (*c*); and YS7212 (*d*). Mice were inoculated s.c. with 5×10^5 B16F10 melanoma cells from culture. Eight days following inoculation of tumor cells, some of the mice were further inoculated with 4×10^6 cfu bacteria (*arrow*). *Data points*, mean for $n = 5$ animals, when 100% of the animals were alive in a group; *bars*, SE. Due to the prolonged survival elicited by YS7212, tumor measurements are not shown after 33 days for animals treated with this strain (···· and *; *d*). YS7212 treated mice survived an average of 55 ± 3 days, with the first tumor reaching 4000 mm^3 by 46 days postinoculation. Similar results were obtained in several repeated experiments.

retardation of tumor growth and prolongation of survival was consistently YS7212 >YS7211>YS7213. YS7212, the most effective strain, produced T/Cs of 1.8–2.2. It is interesting that, although YS7213 selectively amplified within tumors, it was the least effective of the strains with regard to its antitumor activity, possibly correlating with its reduced infectivity toward melanoma cells in culture described above (see "Discussion").

Microscopic Analyses. Tissue samples from a variety of human and mouse tumors infected *in vivo* with YS72 were examined by light microscopy and EM (Fig. 2). Studies were carried out with DBA/2J mice bearing Cloudman S91 melanomas, C57B6 mice bearing B16F10 melanomas, and BALBc *nu/nu* mice bearing human carcinomas of the breast, lung, colon, kidney, and liver. With the light microscope, *Salmonella* positively staining with the Brown-Brenn stain were detected predominantly in and around the margins of necrotic areas of tumors, and individual rods were clearly visible. At the EM level, bacteria were seen in mouse melanomas (Fig. 2*A*) and human carcinomas of the lung (Fig. 2*B*), colon (Fig. 2*C*), and breast (Fig. 2*D*). Not shown are similar EM results with human renal carcinoma CRL1611 and hepatoma HTB52 tumors growing in *nu/nu* mice. In summary of the EM studies, bacteria were found in necrotic areas and in the cytoplasm of tumor cells. Bacteria were often found in apoptotic cells as evidenced by the presence of segmented (karyorrhexic) nuclei (Fig. 2, *C* and *D*). Numerous examples were observed of dividing bacteria within the tumors. Quantitation of the bacteria within the human tumors *in vivo* revealed that, as with B16F10 mouse melanomas (Table 3), 2–4 days after i.p. inoculation of 10^6 cfu, YS72 densities reached remarkable levels of 10^8–10^{10} cfu/g in the human tumors, with tumor:liver ratios reaching as high as 4000:1.

Table 4 *Survival of B16F10 melanoma-bearing C57B6 mice inoculated with Salmonella typhimurium*

Each point represents the mean ± SE for $n = 10$ animals. Mice were administered Baytril antibiotic in the drinking water 2 weeks postinoculation with bacteria and were euthanized when tumors reached 4000 mm^3. The experiments were repeated several times with similar results.

Strain	Mean survival (days ± SE)	T/C
Control (no bacteria)	27 ± 2	1.0
YS7213	36 ± 5	1.3
YS7211	38 ± 10	1.5
YS7212	53 ± 7	2.0

Expression of HSV TK and Prodrug Activation. GCV-mediated inhibition of tumor growth via its phosphorylation by HSV TK has been reported for a number of experimental cancers, including the mouse B16F10 melanoma (25–29). For example, 40–50% reductions in B16 melanoma tumor volume were seen in mice injected intratumorally with either adenovirus or DNA containing the *HSV TK* gene and further treated with GCV at doses ranging from 100 to 150 mg/kg/day for 4–6 days (28, 29). To test the potential of *Salmonella* to deliver therapeutic enzymes to tumors, an HSV TK expression construct with a β-lactamase secretory sequence (plasmid p5-3) was transfected into various strains (Fig. 3). Plasmid-bearing strains were able to target and amplify within tumors, as did plasmid-free strains, and they were also similar to their plasmid-free counterparts in expression of the aerobic hyperinvasive phenotype *in vitro* (data not shown). Immunoblot analyses using an anti-HSV TK mAb (*top*) revealed expression of HSV TK in lysates of wild type and YS72 with p5-3 (*Lanes 2* and *3*) but not in those of 14028 with plasmid but no HSV TK gene (*Lane 1*). In assays of HSV TK phosphotransferase activity (*bottom*), using ^{125}I-dC as a substrate for phosphorylation (21), about 10-fold higher levels of activity were seen in wild-type and YS72 lysates containing HSV TK (*Lanes 2* and *3*) compared to those without HSV TK (*Lane 1*). Similarly, using [^{3}H]GCV as a substrate, kinase activity was readily detected in lysates of strains YS7211/p5-3, YS7212/p5-3, and YS7213/p5-3 expressing HSV TK but not in lysates without HSV TK (data not shown).

Periplasmic Localization of HSV TK. Relatively little enzyme activity was recovered from the culture supernatants (data not shown). However, because immunoblot analyses indicated processing of the secretion signal (Fig. 3), secretion into the periplasmic space of the *Salmonella* was suggested. This was verified by purification of the periplasmic fraction and immunoblot analysis (Fig. 4). In whole-cell preparations, both the precursor form, containing the β-lactamase signal sequence, and the mature processed form, with the signal sequence cleaved, were present. In contrast, the periplasmic fraction

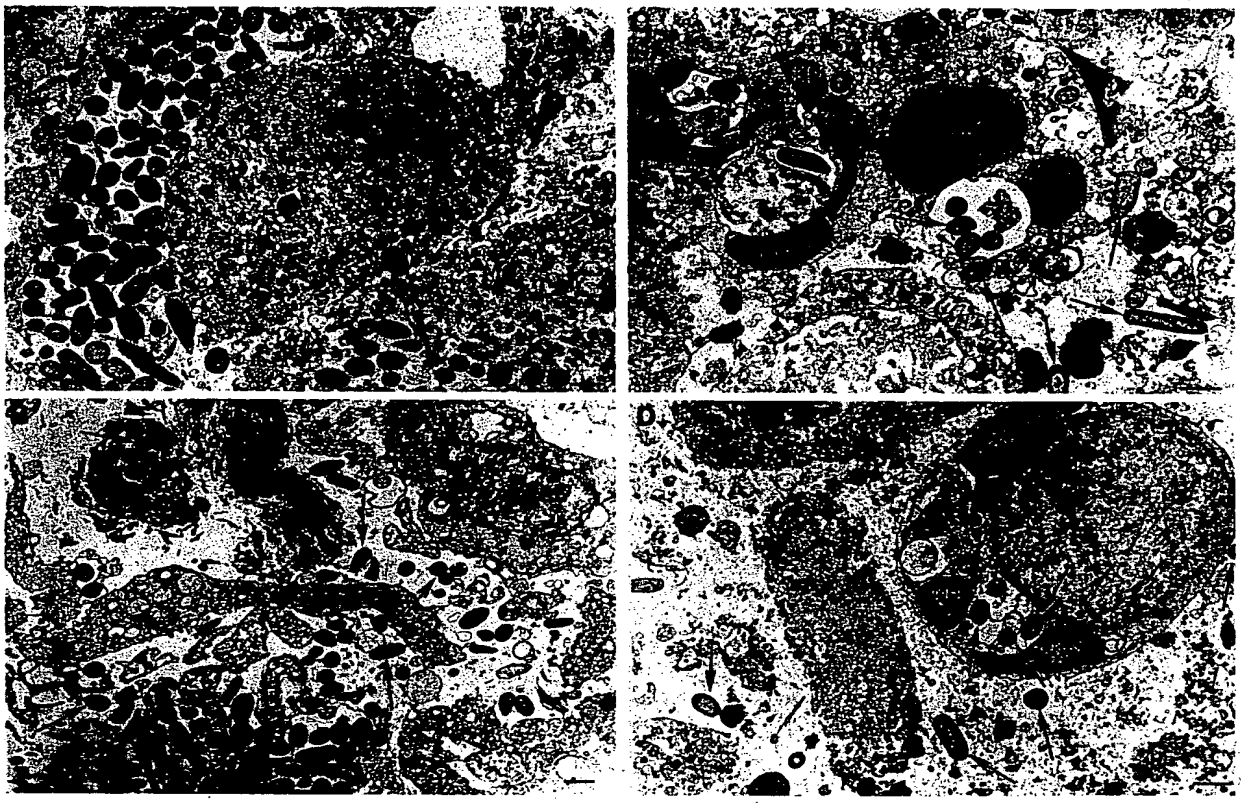

Fig. 2. Electron microscopic analyses of *Salmonella typhimurium* isolate YS72 infecting mouse and human tumors. Procedures were described in "Materials and Methods." *A*, *Salmonella* surrounding and within a B16 melanoma cell located in a necrotic area of the tumor. A single bacterium (*downward arrow*) is seen within the cytoplasm of the melanoma cell along with numerous melanosomes (*m*). A second bacterium (*upward arrow*) is located near the surface of the melanoma cell. Chromosomes (*chr*) are seen in the nucleus of the melanoma cell. *B*, numerous *Salmonella* (*arrows*) in human lung tumor A549. *C*, *Salmonella* in human colon tumor HTC116. Seen is a cell with segmented (karyorrhexic; *kn*) nuclei. Such cells are in late apoptosis (57). *D*, *Salmonella* (*arrows*) in and surrounding a tumor cell from human breast tumor BT20. As in *C*, the tumor cell is in late apoptosis as evidenced by the presence of nuclei in karyorrhexis (*kn*).

contained only the processed form, indicating that processing was necessary for periplasmic localization. Both the precursor and mature forms were seen in the spheroplast preparations, indicating that translocation of the mature form to the periplasm was only partial. As the periplasm is freely permeable to phosphorylated nucleotides, phosphorylated GCV should, in theory, be able pass out of the parasite.

To test the effects of GCV on tumor growth, C57B6 mice were implanted with B16F10 melanoma cells, inoculated 8 days later with strain YS7211 with or without HSV TK-containing plasmid p5-3, and administered GCV as described (Fig. 5). We found that introduction of the plasmid reduced the inherent antitumor activity of the bacteria, such that the plasmid-bearing strains could not be directly compared to the plasmid-free strains for total antitumor activity with and without GCV (see effects of strains without GCV). Nonetheless, a clear effect of GCV-mediated tumor retardation by HSV TK-containing strain YS7211/p5-3 was demonstrated. In control animals 25 days post-tumor implantation, the average tumor volume in mice not receiving bacteria had reached 4000 mm^3, and many of the animals had been euthanized. Control animals not inoculated with bacteria showed small GCV-mediated suppression of tumor growth that was independent of dosage and did not result in enhanced survival. In contrast, tumors in mice treated with YS7211/p5-3 alone had reached only 540 $\pm$ 128 mm^3 (mean $\pm$ SE) by 25 days, whereas those treated with YS7211/p5-3 plus GCV were 193 $\pm$ 59 mm^3 ($P = 0.018$), showing both bacterial as well as GCV effects on tumor growth. On the other hand, without the p5-3 plasmid, GCV showed no effects when used in combination with this strain (day 25, 219 $\pm$ 100 mm^3 *versus* 236 $\pm$ 114 mm^3, control *versus* GCV). For animals treated with YS7211/p5-3, data pooled from three experiments at 28 days post-tumor implantation for all GCV doses from 165 to 330 mg/kg revealed a 2.5-fold reduction in tumor volume by GCV ($P = 0.025$; Table 5). Further, GCV elicited moderate increases in survival (Table 6). Whereas tumor-implanted animals not treated with bacteria died with a mean $\pm$ SE of 25 days without and 27 $\pm$ 1 days with GCV (220 mg/kg), those inoculated with YS7211/p5-3 and no GCV survived 36 $\pm$ 2 days, and those treated with YS7211/p5-3 + GCV survived 40 $\pm$ 3 days for 165 mg/kg ($P = 0.070$), 42 $\pm$ 3 days for 220 mg/kg ($P = 0.085$), and 41 $\pm$ 2 days for 330 mg/kg ($P = 0.023$).

DISCUSSION

Using *Salmonella typhimurium*, we have developed a tumor-amplified protein expression therapy. Our approach involved the combination of a number of mutant characteristics and the transfection of foreign genes to create an attenuated anticancer vector from a highly virulent strain of *Salmonella*. Aerobic hyperinvasiveness was combined with defects in major biosynthetic pathways, including purine, pyrimidine, and amino acid synthesis, to achieve vectors that, when injected from a distant site, accumulated and selectively replicated in tumor tissue. These bacteria had inherent antitumor activity-retarding tumor growth and prolonging average survival to as much as twice that of untreated mice with strain YS7212 (27 days *versus* 53 days, no bacteria *versus* with bacteria). Further, using *Salmonella* expressing the *HSV TK* gene, we showed a GCV-mediated 2.5-fold reduction in B16 melanoma tumor size, a somewhat stronger effect at lower dosages than reported earlier, when B16 tumors were injected intra-

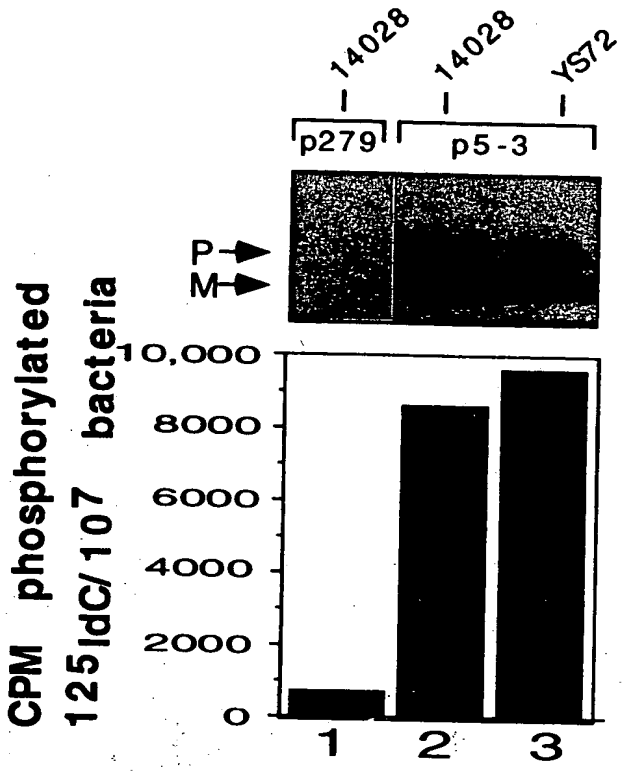

Fig. 3. Detection of HSV TK by immunoblot (*top*) and phosphotransferase activity (*bottom*) assays in bacterial lysates derived from equal numbers of cells of wild-type strains 14028 (*WT*) and YS72. *Top*, immunoblot detection of precursor (*P*) and mature (*M*) forms of HSV TK using an anti-TK mAb. *Lane 1*, wild-type (*WT*) strain 14028 containing plasmid pKT279 without the *HSV TK* gene (p279) showing no reactivity with the anti-TK mAB; *Lanes 2* and *3*, 14028 and YS72, respectively, containing plasmid p5-3 with the β-lactamase secretory sequence fusion form of TK. *Bottom*, phosphotransferase activity associated with each of the samples in the *top*. Activity is expressed as the total number of counts of ^{125}I-dC phosphorylated by lysates derived from 10^7 bacteria (21).

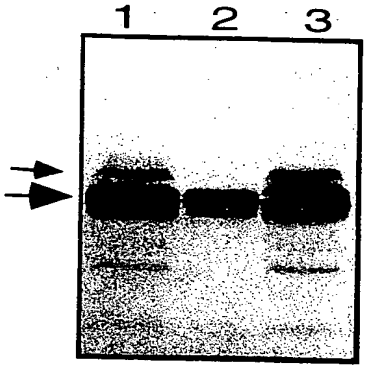

Fig. 4. Periplasmic localization of HSV TK. Immunoblots of whole cell-preparations of bacteria (*Lane 1*), the periplasmic fraction (*Lane 2*), and the spheroplast pellet (*Lane 3*). Whole-cell preparations show the migration of a precursor form (*small arrow*) and the mature form (*large arrow*). The periplasmic fraction shows only the processed form of the protein.

tumorally with DNA or adenovirus containing the *HSV TK* gene (28, 29). Thus, it has been demonstrated that *Salmonella* would be useful both for its inherent antitumor activity and for its ability to deliver therapeutic proteins to cancer cells *in vivo*.

In parallel studies with *Salmonella* in DBA/2J mice bearing Cloudman S91 melanomas, we obtained essentially the same results for selective amplification and tumor retardation as described herein for B16F10 melanomas in C57B6 mice (data not shown). Moreover, in studies with DBA/2J mice, when melanomas were implanted s.c. at four separate sites, and YS72 was inoculated either i.p. or directly into only one of the tumors, selective amplification of YS72 was observed in all four tumors. Selective amplification of YS72 was also seen in spontaneous soft tissue metastases following i.p. inoculation of the bacteria. The results underscore the therapeutic potential of *Salmonella* as a vector for distant metastases as well as for primary tumors (30).

Attenuation of *Salmonella* through auxotrophy reduced their virulence in mice but not their ability to target and amplify 2–3 orders of magnitude in tumors compared to livers. We envision that, because auxotrophic mutants require exogenous nutrients, they are, in general, weakened within the mouse, allowing them to be controlled or eradicated by host defense mechanisms. Tumors, with actively dividing cells as well as necrotic areas, would seem to be an environment in which metabolites such as purines, pyrimidines, and amino acids would be available in good supply, providing a reservoir to the bacteria.

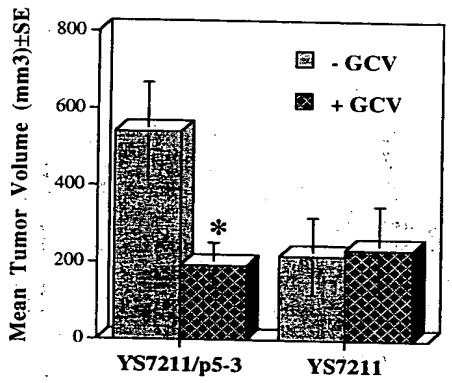

Fig. 5. Effects of GCV on growth of B16F10 tumors in C57B6 mice treated with *Salmonella* strain YS7211 with or without HSV TK-containing plasmid p5-3. Mice were treated with a total of 220 mg/kg GCV as described in "Materials and Methods," and tumor volume (in mm^3) was determined at 25 days post-tumor implantation. *Columns*, mean of pooled data from four separate experiments for $n = 9$–35 animals per point, with 100% survival of treated animals at 25 days; *bars*, SE. *, $P = 0.018$ for difference between strain YS7211/p5-3 with and without GCV.

Table 5 *Effects of ganciclovir on B16F10 tumor growth in C57B6 mice inoculated with Salmonella YS7211 with and without the HSV TK gene*

Results are for mean tumor volume ± SE 28 days post-tumor implantation from pooled experiments for GCV doses of 0 (bacteria only), 165, 220, and 330 mg/kg.

Treatment	Mean tumor volume ± SE
YS7211/p5-3	1377 ± 353 (n = 15/15)
YS7211/p5-3 + GCV	535 ± 102[a] (n = 36/36)

[a] $P = 0.025$.

Table 6. *Effects of GCV on the survival of C57B6 mice inoculated with Salmonella YS7211 ± HSV TK gene*

Results are from pooled experiments with 12–19 animals per group at the start.

Strain	GCV (mg/kg)	Survival (days ± SE)	T/C
Control (no bacteria)	None	25 ± 0	1.0
YS7211	165	27 ± 1	1.1
	0	40 ± 4	1.6
YS7211 + p5-3-2	165	35 ± 4	1.4
	None	36 ± 2	1.4
	55	36 ± 2	1.4
	110	38 ± 2	1.5[a]
	165	40 ± 3	1.6[a]
	220	42 ± 3	1.7[a]
	330	41 ± 2	1.6[a]

[a] See text for *Ps*.

Regarding bacteria and cancer, there are clinical observations dating over more than 200 years in which cancers regressed in patients with bacterial infections (31, 32). At least part of the therapeutic action of bacteria appears to be through stimulation of the host immune system (*e.g.*, Refs. 33–37). However, immunostimulation may not be the only therapeutic mechanism because there are numerous reports of patients with bacterially infected tumors, often containing *Salmonella* (38–41), raising the possibility that bacteria could target tumors and have local antitumor effects.

The mechanisms involved in suppression of tumor growth by *Salmonella* are likely to be multifaceted. Suppression might be due to an activation of the immune system through the release of LPS by the tumor-infecting bacteria. LPS released within the tumor by the infecting *Salmonella* could induce tumor necrosis factor α release by WBC, either systemically or locally, and elicit antitumor effects within the animal (34, 43, 44). LPS from bacteria in the tumor could also serve as an attractant to WBC such as macrophages and neutrophils that, in turn, express antitumor activity. It is also possible that WBC transport *Salmonella* from the circulatory system to tumors, the net result being an increase in both WBC and bacteria within the tumor. Additional explanations for tumor suppression could include competition for nutrients between cancer cells and bacteria, and secretion of bacterial toxins or enzymes such as proteases and other hydrolases.

We do not yet know what role, if any, the aerobic hyperinvasive phenotype of YS72 played in the efficacy of targeting, amplification, or tumor suppression. Evidence that invasion itself may be important comes from our findings that YS7213, noninvasive in culture following either aerobic or anaerobic growth, was also the weakest strain for tumor suppression *in vivo*, although it was capable of targeting tumors and amplifying within them (Table 3). Our microscopic studies of hyperinvasive YS72 indicated that, whereas most of the bacteria were seen in necrotic spaces, they were also detected within the cytoplasm of tumor cells, suggesting that YS72 was capable of invasion of tumor cells *in vivo* (Fig. 2). Perhaps amplification within necrotic spaces, as achieved by YS7213, is sufficient for some tumor suppression; however, more suppression can be gained from bacterial invasion of tumor cells, as apparently observed with YS72. A microscopic study of tumor cell invasion characteristics of YS7213 *in vivo* has not yet been completed.

There were several parameters of the HSV TK/GCV experiments that require discussion and further investigation. We have not measured phosphorylation of GCV or its incorporation into DNA within tumors. Rather, these first studies have used differential effects on tumor growth retardation and prolonged survival to assess its activation. The experiments in mice were complicated by the fact that the bacteria alone were so potent in retarding tumor growth that further GCV effects were relatively small. In addition, the potency of individual *Salmonella* strains alone on tumor growth and survival varied between strains, with YS7212 being more potent than YS7211 and YS7213, and HSV TK plasmid-bearing strains being less potent than their plasmid-free counterparts (Figs. 1 and 5). At least 80% of the bacteria retained the plasmid while infecting tumors 10 days postinoculation, as determined by tetracycline resistance. The differences in tumor suppression between HSV TK plasmid-bearing and plasmid-free strains might have been due to differential virulence; however, this was difficult to determine because each of the three polyauxotrophs was essentially avirulent whether or not it carried the HSV TK plasmid. Whether the plasmid-bearing strains were less virulent than plasmid-free strains was not determined in an LD_{50} study. Plasmid-bearing strains were able to target and amplify within tumors, as did plasmid-free strains *in vivo*, and were also similar to the plasmid-free strains in expression of the aerobic hyperinvasive phenotype *in vitro*. With the inherent difference in tumor suppression between plasmid-bearing and plasmid-free strains, the most meaningful comparisons that support a GCV effect were those that contrasted a single strain without the HSV TK plasmid, showing no effect of GCV, and the same strain with the plasmid, showing an effect of GCV (Fig. 5). With this in mind, YS7211/p5-3 was chosen for the HSV TK/GCV experiments because, when used alone, it was of intermediate antitumor potency compared to the other polyauxotrophs, allowing for further assessment of GCV effects. With this strain, we were able to demonstrate a HSV TK/GCV-dependent retardation of tumor growth and prolongation of survival (Fig. 5 and Tables 5 and 6). Thus, demonstration of GCV activation was achieved, although little is yet known about the underlying mechanisms. Our microscopic studies demonstrated bacteria in great numbers in necrotic areas of tumors, but bacteria also invaded cells of the necrotic margins (Fig. 2). From other studies, we would expect that GCV was phosphorylated by bacteria within these invaded tumor cells, perhaps ultimately being passed through gap junctions to adjacent cells (25–29).

Numerous physical barriers exist in the delivery of therapeutic agents to solid tumors that can compromise the effectiveness not only of drugs but also of antibodies, cytokines, and viruses as therapeutic agents or delivery systems (1). Our results indicate that *Salmonella* have several advantages that overcome such delivery problems. It should also be noted that therapeutic use of live bacteria has been reported previously. For example, in 1947, Parker *et al.* (42) showed that direct injection of spores of *Clostridium histolyticus* into a transplantable sarcoma growing in a mouse caused oncolysis and regression of the tumor. In a study with attenuated Aro$^-$ *Salmonella* and plasmacytoma-bearing mice, i.p. and i.t. injections of bacteria suppressed tumor growth and prolonged survival of mice, with results similar in magnitude to ours reported here for melanoma cells (45). In another *Salmonella* study, an attenuated strain secreting human interleukin-2 decreased hepatic metastases in a murine MCA-38 adenocarcinoma hepatic metastases model (46, 47). In these latter two studies, the effects of the bacteria were attributed to their action as immunostimulatory agents rather than as direct antitumor agents, and tumor targeting of the bacteria was not reported. In other examples, *Salmonella typhimurium* (48–50), *Listeria monocytogenes* (51, 52), *Shigella flexneri* (53), and *Clostridium beijerinck* (54, 55) have been developed for delivery of foreign genes or proteins to mammalian cells *in vitro* and *in vivo*. However, we feel that *Salmonella* has the combined advantages of these systems for tumor therapy. Compared to "attenuated pathogens," including *Salmonella*, *Shigella*, and *Listeria*, in which live organisms are used to deliver therapeutic proteins and antigens, the *Salmonella* described herein represent a significant advance in allowing the direct delivery of therapeutic agents to the target, *i.e.*, malignant tumors. From a perspective of "targeted bacteria," which includes *Clostridia*, genetically modified *Salmonella* are an notable expansion of the field because of the numerous genetic techniques available for the *Enterobacteriaceae*. In other aspects, *Clostridia* only grow in anaerobic tissues, whereas the *Salmonella* developed in this study proliferate and invade mammalian cells under both aerobic and anaerobic conditions, such as found within solid tumors. From the perspective of gene therapy, the ability of *Salmonella* to target multiple tumors from distant sites offers a significant improvement over the many gene therapies limited to local regional administration (*e.g.*, Refs. 27 and 56). With engineered *Salmonella*, a major series of improvements in the bacterium is evident that could result in highly selective targeting and effective prodrug enzyme therapy *in vivo*. Collectively, our findings demonstrate potential new vectors for tumor-targeted cancer therapeutics.

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Antitumor Effect of VNP20009, an Attenuated *Salmonella*, in Murine Tumor Models

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VNP20009, a genetically modified strain of *Salmonella typhimurium* with deletions in the *msbB* and *purI* loci, exhibited antitumor activities when given systemically to tumor-bearing mice. VNP20009 inhibited the growth of subcutaneously implanted B16F10 murine melanoma, and the human tumor xenografts Lox, DLD-1, A549, WiDr, HTB177, and MDA-MB-231. A single intravenous injection of VNP20009, at doses ranging from 1×10^4 to 3×10^6 cfu/mouse, produced tumor growth inhibitions of 57–95%. Tumor volume doubling time, another indicator for tumor growth inhibition, also significantly increased in mice treated with VNP20009. Using mice with immune system deficiencies, we also demonstrated that the antitumor effects of VNP20009 did not depend on the presence of T and B cells. In addition, VNP20009, given intravenously, inhibited the growth of lung metastases in mice. Only live bacteria showed the antitumor effect.

Key words: VNP20009; *Salmonella typhimurium*; Murine tumor model; Antitumor effect

It has long been known that tumor regression occasionally occurs in patients with bacterial infections (1). An infection arising from a tumor may be the first clinical manifestation of neoplastic disease (2). Numerous clinical reports describe patients with tumors infected by bacteria, frequently *Salmonella* (3–6). For example, Giel (4) described an abscess in a pheochromocytoma containing 90 cc of thick, yellow pus encapsulated by a thin sphere consisting of a few layers of tumor cells. The bacteria were later identified as *S. typhimurium.*

The use of bacterial products for cancer treatment dates back to the early 1900s. William B. Coley, who was then a surgeon at Memorial Hospital, now Memorial Sloan-Kettering Cancer Center, observed that patients with sarcoma responded better after surgery if they developed severe postoperative infections. Coley later developed a regimen containing bacterial cell wall components for the treatment of cancer (7). The grain-positive bacterium *Clostridium* was evaluated as an anticancer agent in clinical trials in the 1970s. Although in many cases *Clostridium* was recovered from tumors and oncolysis was observed, these clinical trials were subsequently discontinued because they failed to produce clinical benefits to patients (8). Bacille Calmette-Guerin (BCG[2]), another viable bacterium, is being used for the treatment of superficial bladder carcinoma in humans. BCG, an attenuated, avirulent strain of *Mycobacterium bovis*, is administered by urethral catheterization at periodic intervals for up to 24 months. In patients with bladder cancer, BCG treatment achieves complete responses in greater than 60% of the patients (9).

These reports suggest that bacteria could serve as anticancer agents if their virulence were controlled. *S. typhimurium*, if attenuated, can be safely administered to animals to retard tumor growth in murine tumor models. Avirulent strains of *Salmonella* have been developed as vaccines for the prevention of bacterial infections (10,11). A viable *S. typhi* vaccine, Ty21a, has been approved for the prevention of typhoid fever in humans (12). In addition to serving as vaccines against Salmonellosis, attenuated strains of *Salmonella* have been used for expressing and delivering heterologous proteins to the immune system. This approach may eventually be developed for combating infections and cancer (13,14). Using an *aro4*-mutant SL3225, Eisenstein et al. (14) reported tumor inhibition of a plasmacytoma by either intraperitoneal or intralesional injection of the attenuated bacteria. Similar results have been obtained by Pawelek et al. (15) using other auxotrophic mutants of *S. typhimurium*. To overcome the propensity of grain-negative bacteria to induce septic shock in animals, Low et al. (16) constructed an attenuated *S. typhimurium* by deleting the *msbB* gene, which encodes the enzyme involved in the terminal myristoylation of lipid A. The mutated bacteria lost the capability to induce tumor necrosis factor-α (TNF-α), both in vitro and in vivo, and their pathogenicity was greatly reduced. These bacteria also exhibited the characteristics of preferential accumulation in tumors and inhibition of tumor growth.

We report here that VNP20009, an attenuated strain of *S. typhimurium* with deletions in the *msbB* and *purI* loci, suppresses the growth of subcutaneously implanted tumors and lung metastases. We also show that the antitumor effects of VNP20009 do not depend on the pres-

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[2]Abbreviations used: BCG, Bacille Calmette-Guerin; TNF-α, tumor necrosis factor α; cfu, colony forming unit.

ence of T and B vells, and only live bacteria exhibit the antitumor effect.

MATERIALS AND METHODS

Cell Culture Conditions

All cell lines were maintained at 37 ± 2°C in a humidified atmosphere containing 5% CO_2. B16F10 murine melanoma, DLD-1 human colon carcinoma, A549 human lung carcinoma cells, HTB177 human lung carcinoma cells, Lox human melanoma, and MDA-MB-231 human breast carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. WiDr human colon carcinoma cells were maintained in Eagle's minimum essential medium, containing Earle's salts, and supplemented with both nonessential amino acids and 10% fetal bovine serum.

Tumor Cell Implantation

Solid tumor models were obtained by SC injection of tumor cells in the right hind flank of C57BL/6, athymic nude, or SCID mice. For tumor implantation, cells were detached from the flask by trypsinization, washed, and suspended in PBS to a cell density of 5×10^6 cells/ml (B16F10 cells), 7×10^7 cells/ml (DLD-1 cells), 5×10^7 cells/ml (WiDr, A549, HTB177 cells), and 4.5×10^7 cells/ml (MDA-MB-231 cells). A 0.1 ml bolus of cell suspension, giving a total of 5×10^5 cells (B16F10), 7×10^6 cells (DLD-1), 5×10^6 cells (WiDr, A549, HTB177), or a 0.2 ml bolus, giving a total of 9×10^6 cells (MDA-MB-231), was injected SC into the right flank. Animals were immediately randomized and arranged into groups of 5–10 animals per group. Experimental lung metastases were produced by IV injection of 1.5×10^5 B16F10 murine melanoma cells through the lateral tail vein into C57BL/6 and athymic nude mice. Animals were identified by ear tags.

Treatment of Mice With VNP20009

VNP20009 was grown as a liquid culture to an OD_{600} = 0.8 by suspending a single colony into 25 ml of Luria-Bertani (LB) broth. The suspension was diluted in PBS before use, based on 1 OD = 1×10^9 colony forming unit (cfu)/ml. Solid tumors were staged for 6–24 days prior to bacterial inoculation. A 0.2-ml bolus of the PBS-diluted bacterial suspension was administered IV via the lateral tail vein. Each mouse received approximately 1×10^4 to 3×10^6 cfu of VNP20009. Mice bearing lung metastases were treated with a 0.2-ml bolus of live VNP20009 (containing 2×10^6 cfu) or dead VNP20009 (containing 2×10^8 cfu) 5 days after tumor inoculation. Actual doses were determined by plating the dosing solution on LB agar plates, and colonies were enumerated after overnight incubation at 37°C. Cyclophosphamide (Sigma, Milwaukee, WI), used as a positive control, was given IP at a dosing schedule of 200 mg/kg, once weekly for 3 weeks. In some experiments, SCID mice receiving VNP20009 also received oral ciprofloxacin, 100 mg/kg, twice daily for 5 days. In comparison studies, killed VNP20009 was prepared by autoclaving the bacteria for 20 min, and lack of viability was confirmed by plating.

Tumor Measurements

Tumor size was determined from measurements obtained with electronic calipers along three axes: length (L), width (W), and height (H). The volume of the tumor was calculated using the following formula: tumor volume = $(L \times W \times H)/2$. The mean tumor volume and standard deviation of all animals comprising each group were determined. Tumor volume of individual animals from control and treated groups at the end of experiment was also analyzed with the Student's *t*-test. Lungs with metastases taken from mice were weighed and photographed.

Histopathological Studies

To analyze mechanisms involved in the observed antitumor effects, B16F10 tumors that were treated with PBS or VNP20009 were removed from mice 3–11 days after treatment and fixed with 4% buffered formalin. The specimens were embedded in paraffin, and 6-μm-thick sections were stained sequentially with H&E for microscopic examination.

Animal Care

All animals were treated, fed, housed, and handled according to guidelines established by the National Research Council. The animals used in this study were obtained from Charles River Laboratories (Wilmington, MA). Animals selected in this study were as uniform in age and weight as possible. They were approximately 8–10 weeks of age, and their body weights ranged from 18 to 22 g. Animals were housed in plastic cages with stainless steel covers and were identified by an ear tag. Five mice were housed in each cage. Mouse Chow food was available ad libitum via food hoppers. Tap water was provided in glass bottles ad libitum. All animals were kept in a well-ventilated room where a 12-h light/12-h dark photoperiod was maintained. Room temperature was maintained at 72 ± 2°F. At the end of the study, animals were euthanized in a dry ice-containing chamber.

RESULTS

The ability of VNP20009 to inhibit tumor growth was examined over a dose range of 1×10^4 to 1×10^6 cfu/mouse in the SC B16F10 melanoma model. Mice were administered VNP20009 IV on day 7, and tumor volumes were measured on days 10, 13, 17, and 20 (Fig. 1). The differences observed between individual groups were deemed significant when analyzed by the Student's two-tailed *t*-test. All doses were found to give significant antitumor activity ($P < 0.01$). A single dose of VNP20009 at 1×10^5 cfu/mouse produced responses equivalent to, or better than, the antitumor drug cyclophosphamide, which was given three times during the course of treatment.

Figure 2 demonstrates that VNP20009 inhibited the growth of B16F10 melanoma implanted into immunocompetent C57BL/6 (Fig. 2A), athymic nude (Fig. 2B), or SCID mice (Fig. 2C). A single injection of VNP20009 produced a tumor growth inhibition of 85% or more re-

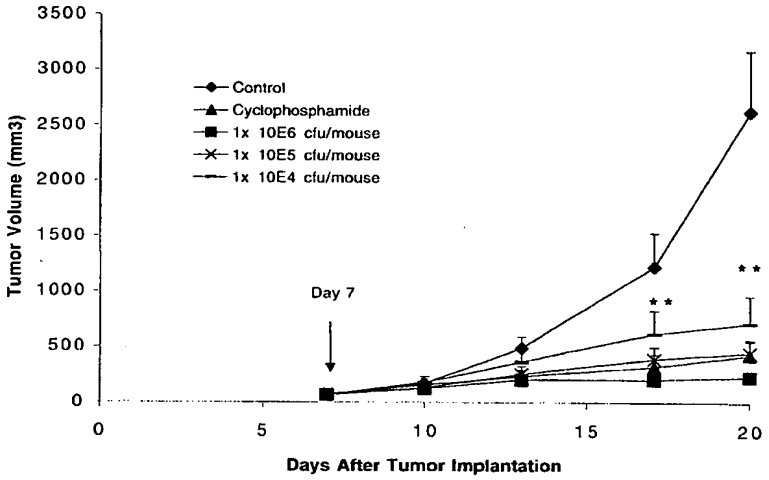

Figure 1. Tumor growth inhibition of B16F10 melanoma by VNP20009. A single dose of VNP20009 (from 1×10^4 to 1×10^6 cfu/mouse) was injected IV into mice 7 days after tumor implantation. Cyclophosphamide was given IP at 200 mpk on days 7, 14, and 21. $^{**}P < 0.01$).

gardless of strain of mice used. SCID mice were sensitive to VNP20009 treatment, and started dying 7 days after bacterial injection when no antibiotics were given. No mortality or weight loss was noted in C57B/6 or athymic nude mice treated with VNP20009. Significant antitumor activities ($P < 0.01$, t-test) were recorded in all the treated groups.

Athymic nude mice bearing A549, WiDr, or DLD-1 tumors were treated with VNP20009 at a dose of 2×10^6 cfu/mouse on day 6 (A549), day 8 (WiDr), or day 13 (DLD-1). A single injection of VNP20009 caused significant and sustained tumor growth inhibitions of 54%, 65%, and 74% for A549, WiDr, and DLD-1, respectively (Fig. 3). MDA-MB-231 tumors grew in nude mice and were staged for 24 days before dosing. Similar results were obtained, with a single dose of VNP20009 inhibiting the growth of MDA-MB-231 tumors more than 70%. HTB177 lung carcinoma staged to 11 days after implantation into SCID mice was treated with a dose of 1×10^6 cfu/mouse. This dose of VNP20009 produced a 94% tumor growth inhibition at the 10-day interval (Fig. 3E). The experiment was terminated on day 20 because of the death of animals caused by bacterial infection. VNP20009 inhibited tumor growth regardless of size of tumors at the time of treatment (0.05–0.3 g). It was equally efficacious in inhibiting fast (B16F10, Lox) or slow growing (MDA-MB-231, DLD-1) tumors.

In another experiment using SCID mice bearing Lox human melanoma, the antibiotic ciprofloxacin was given to mice receiving VNP20009. All the SCID mice treated with VNP20009 alone died before day 15 (i.e., 10 days after VNP20009 dosing). In contrast, all the mice survived that received ciprofloxacin 4 days after VNP20009 treatment. In addition, a very significant delay of tumor growth was observed in animals receiving VNP20009 alone or VNP20009 plus ciprofloxacin (Fig. 3F). Tumor volume doubling time in treated animals was significantly increased compared with vehicle-treated controls.

VNP20009, at a dose of 2×10^6 cfu/mouse injected IV, inhibited the growth of lung metastases compared with untreated controls (Fig. 4). Similar experiments carried out in athymic nude, SCID, and beige mice produced comparable results. In addition, only viable bacteria inhibited the growth of lung metastases. When VNP20009 was heat inactivated, even if given at a higher amount, no antitumnor effects were observed (Fig. 5).

Tumor sections were prepared from untreated mice and mice treated with VNP20009 for various days. At least three tumors for each time point and 20 slides from each tumor were examined for the presence or absence of necrosis and infiltrating cells. Sections taken from tumors treated with VNP20009 revealed a massive infiltration of immune cells accompanied with an extensive necrosis in the central part of the tumors. Significant tumor necrosis (over 70% surface of tumor tissue) was found in mice treated with VNP20009 for 7 days, compared with PBS-treated control mice (Fig. 6). The dead and dying tumor cells were stained with bright red color, characterized with an increase in cell size and the disintegration of the nuclei. Infiltrating cells formed a zone surrounding the necrotic center of the tumor (Fig. 6A). Most of infiltrating cells, at higher magnification, were shown to be neutrophils with horseshoe-shaped nuclei (Fig. 6B). In contrast, tumors obtained from mice treated with PBS (Fig. 6C) revealed a relatively low level of necrosis at the center (less than 5% surface of tumor section) and with only a few infiltrating immune cells (Fig. 6D).

DISCUSSION

In previous studies, we have demonstrated that a single dose of YS1629, a *msbB* knockout mutant, is highly effective in inhibiting the growth of B16 murine mela-

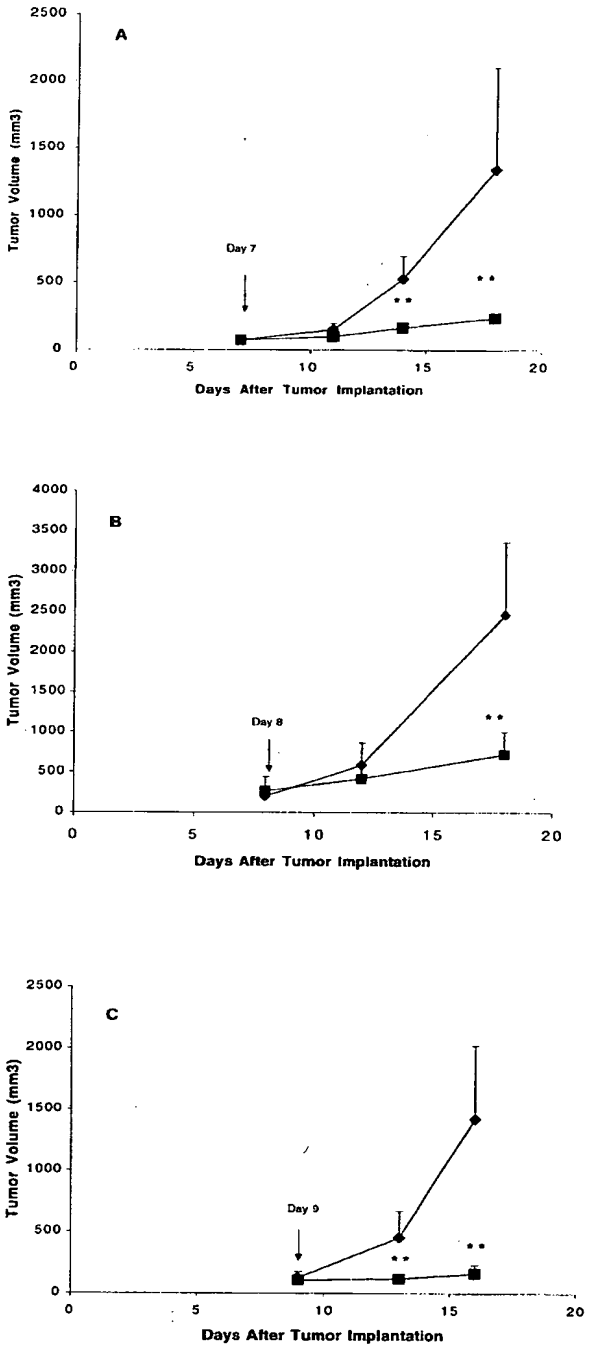

Figure 2. Antitumor effect of VNP20009 on B16F10 melanoma implanted in immunocompetent C57BL/6 (A), athymic nude (B), and SCID mice (C). VNP20009, at a dose of 2×10^6 cfu/mouse was injected IV into tumor-bearing mice 7–9 days after tumor implantation. $^{**}P < 0.01$.

noma (15). In this report, we further demonstrate that a single dose of VNP20009, a tetracycline-sensitive, *msbB* and *purI* knockout mutant, is also capable of suppressing tumor growth in human tumor xenograft models representing melanoma and lung, breast, and colon cancer. Similar to previous observations with B16F10 melanoma, a single dose of VNP20009 inhibited tumor growth, consistent with an ability of VNP20009 to persist within tumor xenografts (17). Only a slight dose–response correlation was observed between VNP20009 levels and tumor inhibition in B16F10 melanoma, because VNP20009 replicates within the tumor, producing a greater than expected potency at lower doses (18). The antitumor activity of VNP20009, at the dose of 1×10^5 cfu/mouse, is superior to that induced by the antitumor drug cyclophosphamide, which has been given at the optimal dosing schedule for this tumor.

Toxicology study of the VNP20009 revealed that SCID mice were approximately 5- to 50-fold less tolerant compared with immune-competent mice (data not shown), which was consistent with the enhanced sensitivity of SCID mice to *Salmonella* infection previously reported with other attenuated *Salmonella* strains (19). Athymic CD-1 nude mice and immune-competent CD-1 mice had similar LD_{50} values for VNP20009 (data not shown). These data suggest that B cells may be involved in the clearance of VNP20009. Without receiving treatment of ciprofloxacin, SCID mice succumbed to bacterial infections and began to die 10 days after VNP20009 dosing. Nevertheless, VNP20009-mediated mortality in SCID mice could be completely prevented by antibiotic administration, without affecting the antitumor efficacy. Number of bacteria in both liver and tumor decreased by 90% to 99% with the current dosing schedule during antibiotic treatment but returned to normal levels a few days after treatment ended (data not shown). Page-Clisson et al. (20) reported similar results that ciprofloxacin could not completely eradicate the persistence of *S. typhimurium* in liver and spleen. It should also be noted that VNP20009 inhibited tumor growth in immunocompromised mice with T-cell and B-cell deficiencies. Using an experimental metastasis model, we demonstrated that the antitumor activity of VNP20009 may not require T cells, B cells, and natural killer cells (unpublished data). Schafer et al. (21) reported that live, attenuated *Salmonella* was capable of inducing tumoricidal macrophages in C3H/HeJ mice, whereas BCG was not effective in that strain of mice. Our finding that only viable VNP20009 shows antitumor effects suggests that the antitumor mechanism of VNP20009 differs from that of BCG.

The mechanisms involved in tumor growth inhibition by VNP20009 are not completely understood. It is possible that some cell wall components of live *S. typhimurium* are cytotoxic to mammalian cells and induce apoptosis in macrophages and granulocytes (22). Galan and his coworkers suggested that a type III secretion system is responsible for the *Salmonella*-induced apoptosis in cultured mammalian cells (23). VNP20009 accumulated at high levels inside the tumor mass, which could induce low levels of cytokines, such as TNF-α in situ that elic-

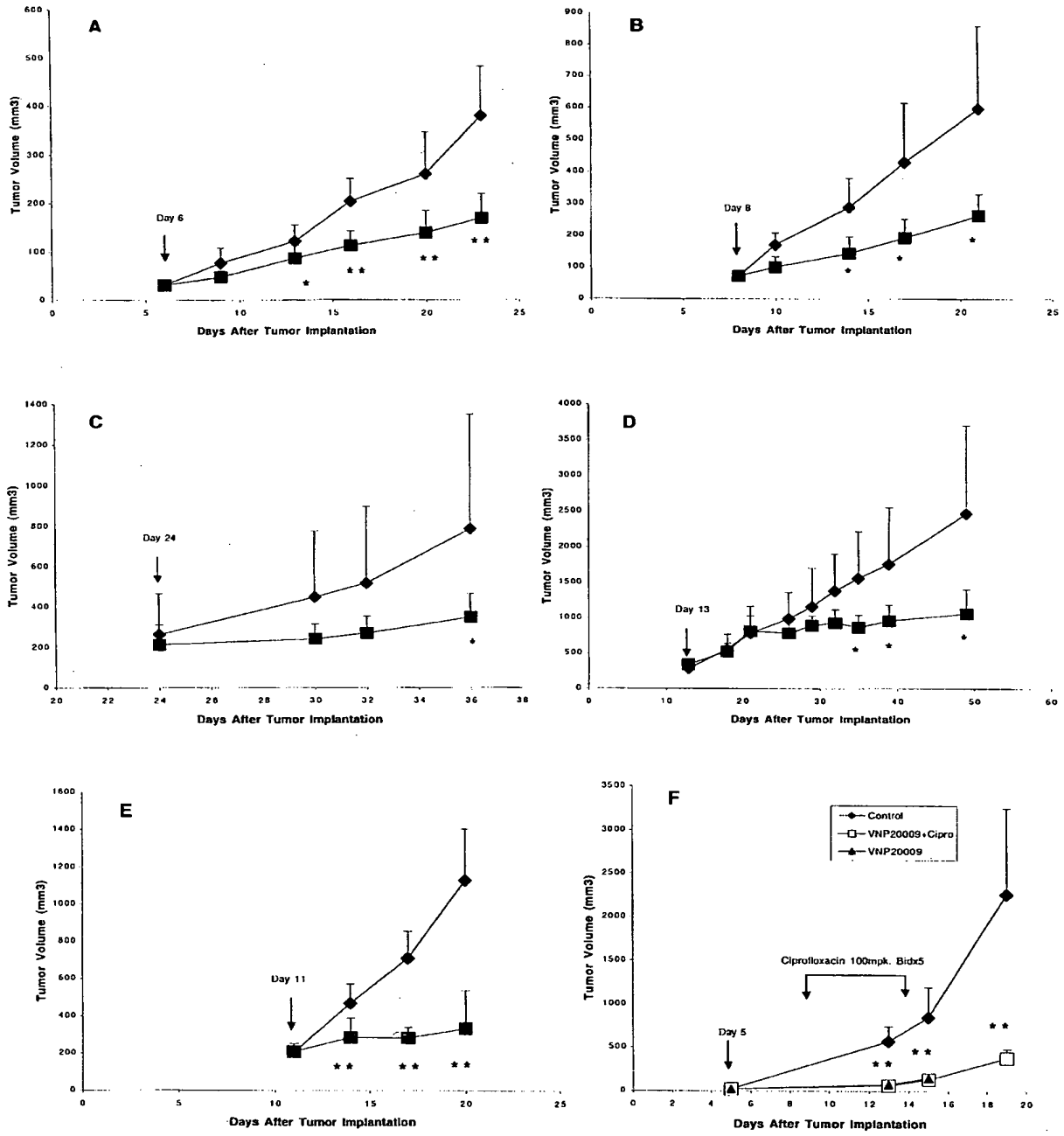

Figure 3. Antitumor effect of VNP20009 on human tumor xenografts. (A) A549, (B) WiDr, (C) MDA-MD-231, (D) DLD-1, (E) HTB177, and (F) Lox cells implanted into either athymic or SCID mice. VNP20009 was given IV at doses ranging from 1×10^6 to 3×10^6 cfu/mouse 6 to 24 days after tumor implantation. $^*P < 0.05$; $^{**}P < 0.01$.

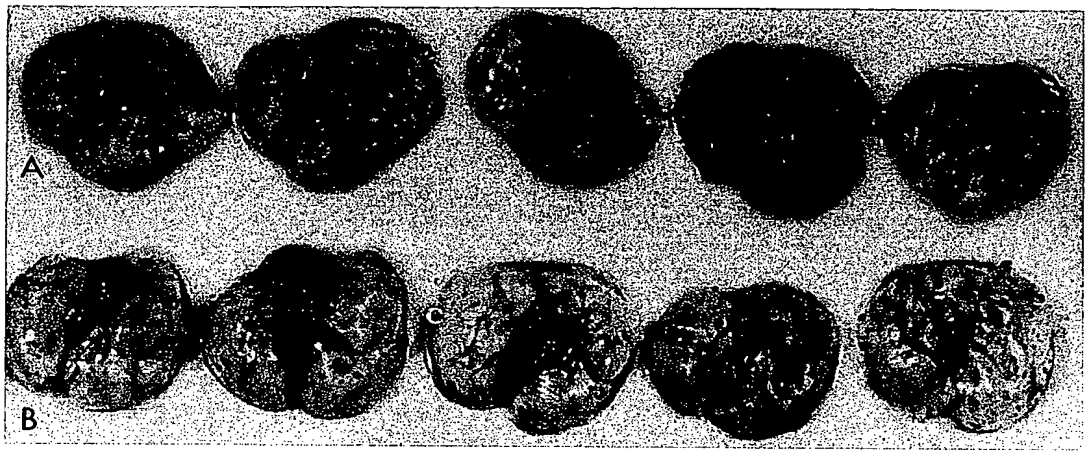

Figure 4. Growth inhibition of lung metastases by VNP20009. A single IV injection of VNP20009 at 2×10^6 cfu/mouse was given to C57BL/6 mice 5 days after inoculation of B16F10 cells. (A) PBS control, (B) VNP20009 treatment.

its an antitumor activity. Other possible mechanisms may include competition between bacteria and tumor cells for nutrients and oxygen. Histolgic evaluation revealed the presence of VNP20009 in both the necrotic center and peripheral portion of the tumor (17). We have further demonstrated in this study that significant tumor necrosis occurred in treated but not untreated mice. In the treated mice, tumor necrosis was also accompanied with a massive infiltration of immune cells including neutrophils. The contribution of these factors to the antitumor activity of VNP20009 is unknown and remains to be elucidated.

Unlike Clostridium, which is an obligate anaerobe and colonized only in hypoxic and necrotic areas of tumors, *S. typhimurium* is a facultative anaerobe and is distributed homogeneously throughout the entire tumor and is accumulated in tumors as small as 0.1 g (24). *Clostridium* accumulated and exerted its oncolytic effects only in large tumors (8,25,26); tumor size ranging from approximately 0.7 g (26) to 1.4 g (25) was needed to allow germination of the bacteria and the subsequent lysis of tumors. Using Sarcoma 180 SC implanted in Taconic Farms Swiss female mice, Thiele et al. (25) reported that tumors of 0.6 g and below were less efficacious in accumulating *Clostridium*. In contrast, we demonstrate here that VNP20009 has antitumor activity against small tumors, with tumor size ranging from 0.05 to 0.3 g at the time of treatment in various murine tumor models. VNP20009 accumulated efficiently in SC implanted B16F10 melanomas with size 1 g or above but antitumor activity has not been evaluated under these conditions. VNP20009 has been attenuated at least 10,000-fold compared with the activity of the parental wild-type *Salmonella* strain in immunocompetent C57BL/6 mice (27). Our studies suggest that T-cell-deficient nude mice are not overly sensitive to VNP20009, in contrast to studies using mice with both T-cell and B-cell deficiency. These results are consistent with a previous report demonstrating that moderate immunodeficiency, resulting from sublethal irradiation of BALB/c mice, does not cause an increase in susceptibility to an attenuated *aro4*-strain of *Salmonella* (28). VNP20009 is well

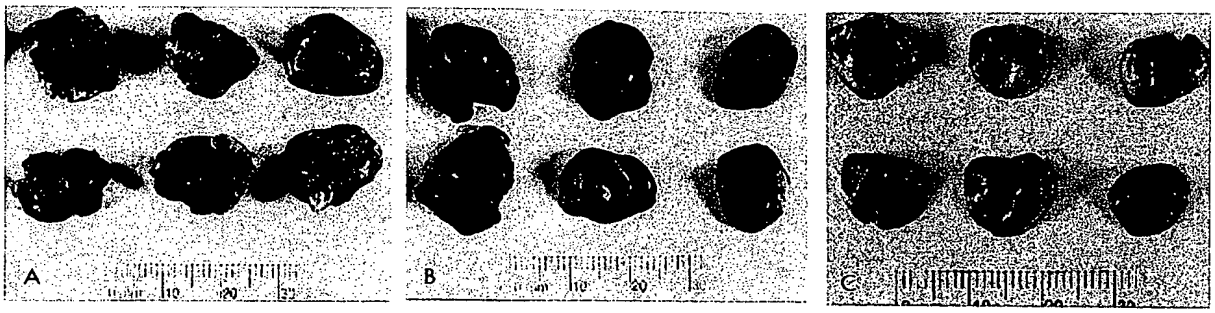

Figure 5. Tumor growth inhibition of lung metastases by live or dead VNP20009. A single IV injection of live VNP20009 at 2×10^6 cfu/mouse or dead VNP20009 at 2×10^8 was given to athymic nude mice 5 days after inoculation of B16F10 cells. (A) PBS control, (B) dead VNP20009 treatment, and (C) live VNP20009 treatment.

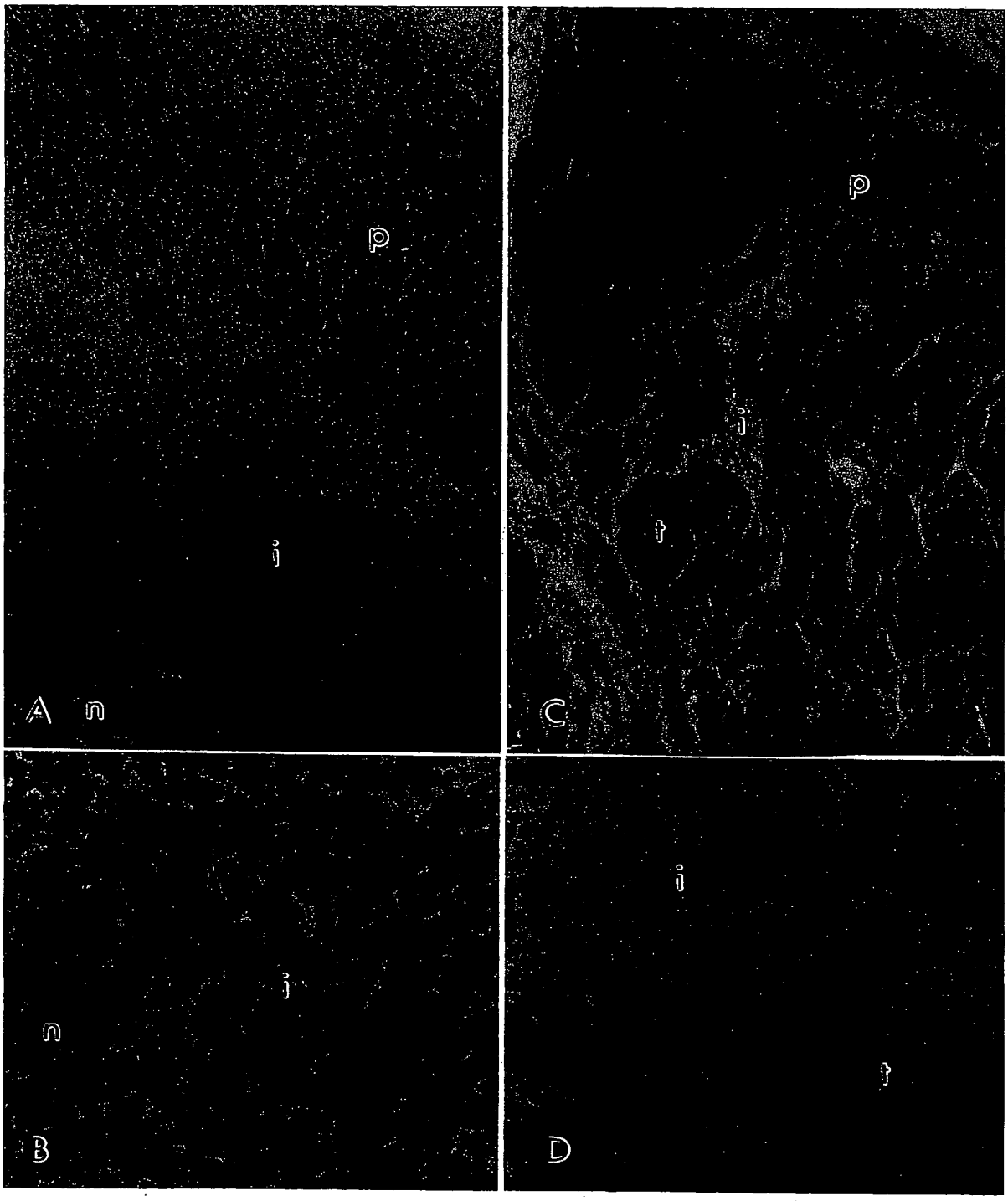

Figure 6. Histologic evaluation of VNP20009 in murine B16F10 melanoma. At low magnification (×100) of VNP20009-treated group (A), tumor was taken from mice treated with VNP20009 at 18 days after tumor implantation and 7 days after bacterial treatment. The periphery of the tumor (p) consisted with tumor cells, host fibroblasts, and infiltrating cells. A layer of infiltrating cells (i) surrounded the dead and dying tumor cells (red color), which represents the necrotic center of the tumor (n). At higher magnification (×400) of VNP20009-treated group (B), most of infiltrating cells were shown to possess horseshoe-shaped nuclei and were identified as neutrophils. In PBS-treated control mice (C), very few necrotic cells (×100) and infiltrating cells (D) were found in the tumor tissue (t) (×400).

tolerated in nonhuman primates; the highest nontoxic dose via IV injection is 2.5×10^8 cfu/kg (18,27). The probability of VNP20009 reverting to wild-type bacteria is unlikely, because VNP20009 was attenuated by deleting both the *msbB* and *purI* genes from the bacterial genome. Because of its potent inhibitory effect in a wide spectrum of human tumor xenografts and its relatively nontoxic characteristics in nonhuman primates, VNP20009 is currently being evaluated as a potential antitumor agent in humans. Moreover, VNP20009 preferentially accumulated in tumor tissues, reaching levels as high as 1×10^9 cfu/g tumor, a concentration 1000 times higher than that found in liver and other normal tissues (17). The use of VNP20009 as a tumor-selective vector to deliver cytosine deaminase to tumors is currently being evaluated in human clinical trials (29).

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Pawelek et al.

Application No.: 09/358,052

Group Art Unit: 1636

Filed: July 21, 1999

Examiner: Sandals, W.

For: VECTORS FOR THE DIAGNOSIS AND TREATMENT OF SOLID TUMORS INCLUDING MELANOMA

Attorney Docket No.: 8002-051

DECLARATION OF DR. DAVID BERMUDES UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, DAVID BERMUDES, do declare and state that:

1. I am a resident of the United States, residing at 524 N. Main Street, Wallingford, Connecticut, 06492.

2. I presently hold the position of Director of Microbiology at Vion Pharmaceuticals, Inc., which position I have held since 1999. I am also currently an Assistant Professor, Adjunct, Department of Internal Medicine, Yale University School of Medicine (1996-Present). I held the positions of Associate Director of Biology at Vion Pharmaceuticals, Inc. from 1997-1999; Senior Research Scientist at Vion Pharmaceuticals, Inc. from 1993-1995; Associate Research Scientist, Department of Internal Medicine, Yale University School of Medicine from 1993-1995; post doctoral associate at Yale University from 1991-1993; and post doctoral associate at Medical College of Wisconsin from 1989-1991.

3. I received the degree of Doctor of Philosophy in Cell and Molecular Biology from Boston University in 1987; and a BA in Biology from Oberlin College in 1982.

4. I am a co-inventor of the present application. I have read and am familiar with the specification of the above-identified application. I have been informed that claims of the above-identified patent application relating to use of tumor-targeted microorganisms are subject to a rejection based on lack of enablement because it is alleged that the specification does not enable practice of the claimed methods with "non-*Salmonella*" microorganisms.

5. The following experiments were conducted in my laboratory, by me or under my supervision, in accordance with the teaching of the specification of the present application. In the experiments described in the following paragraphs, three different non-*Salmonella* microorganisms were used, including a strain of gram-negative *Shigella* species, *i.e.*, *Shigella flexneri* which functions under both aerobic and anaerobic conditions, *Listeria monocytogenes* (ATCC strain 43251), a gram-positive species which functions under aerobic and anaerobic conditions, and a gram-positive *Streptococcus* species, *i.e.*, *Streptococcus agalactiae* (ATCC strain 13813) which functions under both aerobic and anaerobic conditions. The experiments were conducted following the teachings of the specification and basic microbiology techniques known to individuals of ordinary skill in the art. The first set of experiments described below demonstrate that the three non-*Salmonella* microorganisms tested have tumor-targeting abilities. The second set of experiments described below demonstrate that non-*Salmonella* tumor-specific microorganisms investigated have tumor-inhibiting ability when administered *in vivo*.

6. The following two paragraphs describe how illustrative non-*Salmonella* microorganisms were cultured prior to determining their tumor-targeting and/or tumor-inhibiting abilities.

6.1. A *Shigella* species, *i.e.*, *Shigella flexneri* ("*Shigella*") and *Listeria monocytogenes* ("*Listeria*") were grown at 37°C either in Luria broth (LB) liquid media with shaking at approximately 225 revolutions per minute, or on LB solid media containing 1.5%

agar. LB consisted of 10 g tryptone, 5 g yeast extract and 10 g NaCl per liter. The pH of LB was adjusted to 7 using a 1N solution of NaOH. Generally, cultures were streaked out on solid media and incubated until visible growth was observed, and then introduced into liquid media and grown to an appropriate density before freezing in 15% glycerol at -80°C. *Shigella* was grown to an OD_{600} of 1.9, corresponding to 1.03×10^9 c.f.u./ml. *Listeria* was grown to an OD_{600} of 0.6, corresponding to 0.98×10^8 c.f.u./ml.

6.2. A *Streptococcus* species, *i.e.*, *Streptococcus agalactiae* ("*Streptococcus*") was grown in heart brain infusion ("BHI"; Difco) liquid media or on BHI solid media according to the methods described in ¶ 6.1, *supra*. *Streptococcus* was grown to an OD_{600} of 0.85, corresponding to an estimated 2.5×10^8 c.f.u./ml. The bacterial culture was used without freezing for tumor-targeting experiments (¶ 7.2 below). For tumor inhibition experiments (¶ 8.2 below), *Streptococcus* colonies were inoculated into liquid culture as described above, grown to an OD_{600} of 1.2, and frozen at -80°C in 15% glycerol, corresponding to 1.2×10^8 c.f.u./ml after thawing.

7. Non-*Salmonella* Genera Target Selectively to Solid Tumors

7.1. The experiments described below, conducted using methods taught in the specification (*see, e.g.*, Section10.2 at page 72 and Section 23 at pages 133-134) and exemplified therein using *Salmonella*, demonstrate that *Shigella*, *Listeria*, and *Streptococcus* preferentially replicate in tumors when administered *in vivo*.

7.2. To determine the tumor-targeting capabilities of *Shigella*, *Listeria* and *Streptococcus*, the microorganisms were administered to mice having established melanomas, and the ratio of the concentration of microorganisms in the tumor to the concentration of microorganisms in the liver in each mouse was determined as detailed below The method of determining the preference of an organism for tumor versus non-tumor tissue employed is essentially as illustrated in the specification using *Salmonella typhimurium* (*see, e.g.*, Section 10.2, page 72) and *Listeria monocytogenes* (Section 23, pages 133-134).

7.2.1. The mice were implanted with B16F10 murine melanoma tumor cells and the microorganisms administered when the tumors weighed approximately 1g, or approximately after 14-16 days post tumor cell implantation. The use of B16F10 murine melanoma tumor cells is described in the specification, as is a period of tumor cell growth prior to administration of the tumor-specific microorganisms similar to that used in the *Shigella, Listeria* and *Streptococcus* targeting assays, for example at page 95, line 19-23 of the specification.

7.2.2. Prior to administration, frozen *Shigella* and *Listeria* cultures were thawed at room temperature and diluted into phosphate buffered saline (PBS). For *Streptococcus* administration, a fresh culture was diluted into PBS. The microorganisms were administered intravenously into the mice in the following amounts: 2×10^6 c.f.u. of *Shigella*; 1×10^4 or 1×10^5 c.f.u. of *Listeria*; and 1×10^5 or 1×10^6 c.f.u. of *Streptococcus*. The amounts of microorganisms administered are similar to the experimental ranges exemplified in the specification for *Salmonella*. For example, Section 10.3.1 describes the administration of 7×10^4 and 7×10^5 c.f.u. of *Salmonella* per mouse at page 74, lines 9-10; Section 13.5 describes the administration of 1×10^6 c.f.u. of *Salmonella* per mouse at page 89, line 17; Section 15.1 describes the administration of 3×10^5 c.f.u. of *Salmonella* per mouse at page 93, line 29 and 3.6×10^6 c.f.u. of *Salmonella* per mouse at page 94, line 19; Section 15.2 describes the administration of 1×10^5 c.f.u. of *Salmonella* per mouse at page 95, line 23.

7.2.3. At day 5 following administration of the microorganisms, the mice were sacrificed and tumors and livers harvested and homogenized. Serial dilutions of the homogenates were then plated to the appropriate media for each species. These experiments are analogous to experiments described in the specification, *inter alia*, at page 75, lines 22-25, and page 109, lines 12-17.

7.2.4. No undue experimentation was required to identify suitable parameters for the tumor-targeting assay or for quantifying the amount of microorganisms in mouse tissues.

7.3. The results of these experiments are shown in Table I, attached as Exhibit 1. These data indicate that these gram positive and gram negative bacteria, which function under both aerobic and anaerobic conditions, have highly significant targeting ratios for tumors as compared to normal tissues (liver), demonstrating their suitability as tumor-specific vectors as defined in the specification at page 17, lines 22-28.

8. Administration of Non-*Salmonella* Genera Reduces Volume or Inhibits Growth of Solid Tumors

8.1. The experiments described below, conducted using methods taught in the specification, *inter alia* at page 27, lines 7-11, page 54, lines 29-34, and page 122, line 23 through page 24, line 7, demonstrate that *Shigella*, *Listeria*, and *Streptococcus* are capable of inhibiting tumor growth *in vivo*. The results further demonstrate that following the teachings of the specification, the methods for using microorganismal species of the invention for reducing volume or inhibiting growth of a solid tumor cancer can be practiced by one of ordinary skill in the art without undue experimentation.

8.2. To determine the ability of the tumor-targeting *Shigella*, *Listeria* and *Streptococcus* to inhibit tumor growth, the effect of administration of the tumor-specific microorganisms on the growth of established melanomas was determined according to methods exemplified in the specification using *Salmonella typhimurium* (*see, e.g.*, Section 16.1, page 99 and Section 16.2, page 103). The use of antibiotics in conjunction with the tumor-specific microorganisms is also exemplified in the specification using *Salmonella typhimurium* (*see, e.g.*, Section 11.2, pages 77-78 and Section 18.8, pages 122-124).

8.2.1. Mice were implanted subcutaneously with B16F10 murine melanoma tumor cells (5×10^5 cells per animal). The *Shigella*, *Listeria* or *Streptococcus* were administered intravenously when the tumors weighed approximately 0.3 g. Frozen stock of the microorganisms (See, ¶¶ 6.1 and 6.2 above) were thawed at room temperature and diluted into PBS. The microorganisms were administered intravenously into the mice in the following amounts: 2×10^6 c.f.u. of *Shigella*; 1×10^5 c.f.u. of *Listeria*; and 1×10^6 c.f.u. of *Streptococcus*.

8.2.2. Following the first week of administration of the tumor-specific microorganisms, two doses (one on day 5, one on day 8 post-bacteria) of 500 mg ampicillin/kg body weight were administered to the mice that had received *Listeria*. The administration of antibiotics to mice treated with non-attenuated strains of microorganisms for the alleviation of systemic effects of the microorganisms is described in the specification, for example at page 92, lines 9-15 and page 103, lines 19-23.

8.3. Tumor volume was monitored approximately every 5 days. Tumor growth is graphically depicted as tumor volume versus time in Figure 1, attached as Exhibit 2. Tumor growth in mice receiving the tumor targeted *Listeria, Shigella* or *Streptococcus* was inhibited by at least approximately 40% relative to tumor growth in control animals, and up to approximately 65% in the case of *Listeria*. These data indicate that these facultative, gram positive and gram negative bacteria reduce tumor volume or inhibit tumor growth.

9. Based on the experiments described above I conclude, and I believe one of ordinary skill in the art would also conclude, that the teachings of the specification are applicable to non-*Salmonella* microorganisms. Therefore the specification provides sufficient guidance to identify tumor-specific non-*Salmonella* microorganisms and to administer the tumor-specific non-*Salmonella* microorganisms to a subject to inhibit tumor growth or reduce tumor volume. Accordingly, I conclude, and I believe one of ordinary skill in the art would also conclude, that the claimed invention can be practiced without undue experimentation by following the teachings of the specification.

10. In view of the foregoing, I conclude, and it is my considered scientific opinion, that the use of the tumor-specific microorganisms, including but not limited to non-*Salmonella* genera of microorganisms, for reducing volume or inhibiting growth of a tumor, can be practiced according to the methods described in the present application by one of ordinary skill in the art without undue experimentation.

11. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements and the like so made are punishable by fine or

imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the specification or any patent issuing thereon.

Dated: Sept 8, 2000

David Bermudes, Ph.D.

Attachments:

Exhibit 1: Table I

Exhibit 2: Figure 1

Strain	Dose	Tumor c.f.u./g (mean ± SE)	Liver c.f.u./ (mean ± SE)	Ratio
Shigella	2×10^6	$6.7 \pm 5.8 \times 10^7$	$5.5 \pm 5.8 \times 10^2$	120,000:1
Listeria	1×10^4	$2.7 \pm 2.4 \times 10^8$	$7.3 \pm 6.9 \times 10^5$	370:1
Listeria	1×10^5	$2.3 \pm 0.8 \times 10^8$	$3.1 \pm 1.9 \times 10^6$	70:1
Streptococcus	1×10^5	$6.1 \pm 2.9 \times 10^8$	$2.4 \pm 2.4 \times 10^3$	250,000:1
Streptococcus	1×10^6	$3.0 \pm 1.6 \times 10^9$	$2.5 \pm 2.5 \times 10^2$	12,000,000:1

Table I. Tumor to normal tissue (liver) relative accumulations of *Shigella*, *Listeria*, and *Streptococcus*. Counts are based on colony forming units (c.f.u.) and given as the mean ± standard error (SE).

Anti-tumor Effect of Streptococcus, Listeria and Shigella

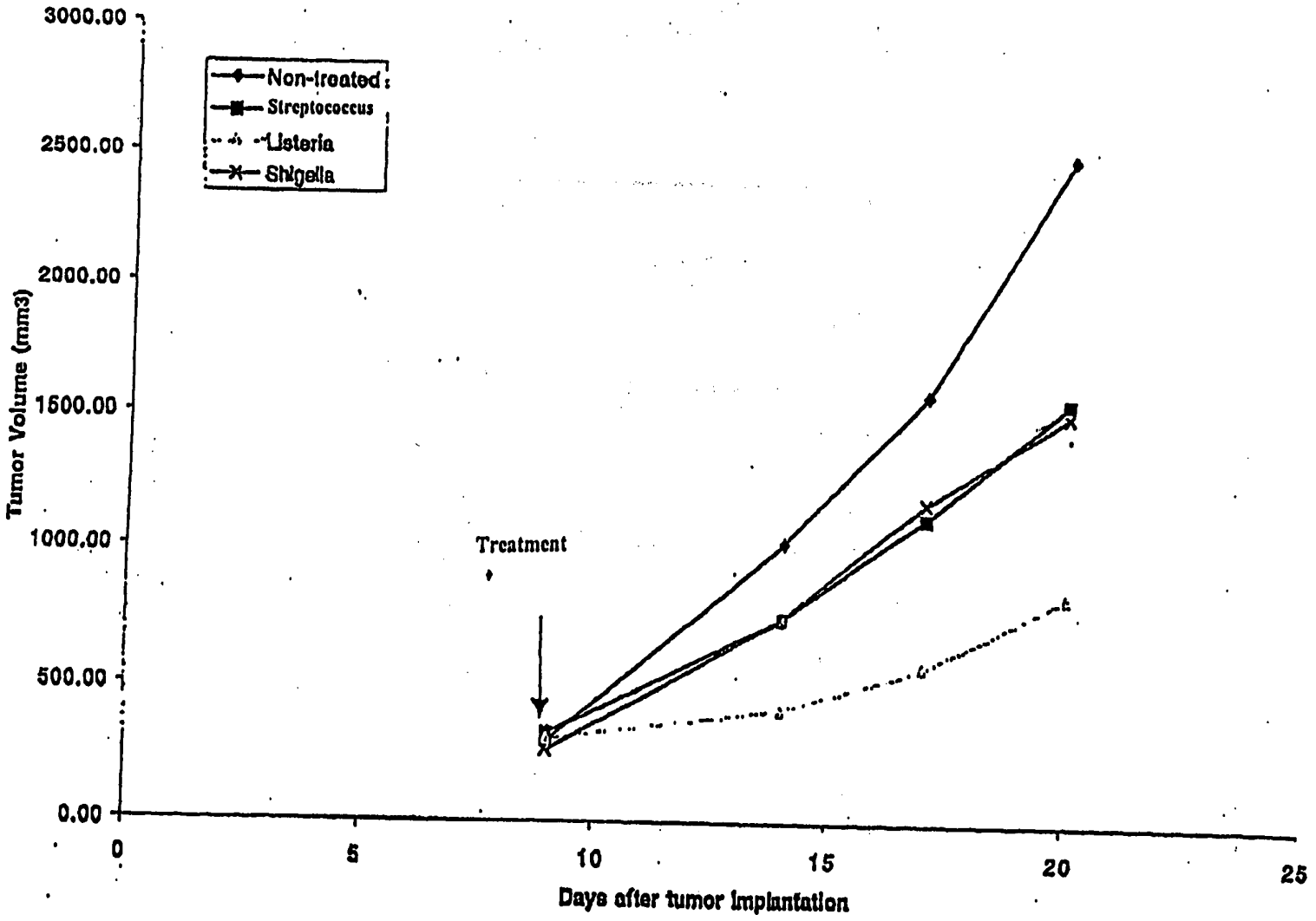

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FIGURE 1

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Visualization of tumors and metastases in live animals with bacteria and vaccinia virus encoding light-emitting proteins

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We have shown that bacteria injected intravenously into live animals entered and replicated in solid tumors and metastases. The tumor-specific amplification process was visualized in real time using luciferase-catalyzed luminescence and green fluorescent protein fluorescence, which revealed the locations of the tumors and metastases. *Escherichia coli* and three attenuated pathogens (*Vibrio cholerae*, *Salmonella typhimurium*, and *Listeria monocytogenes*) all entered tumors and replicated. Similarly, the cytosolic vaccinia virus also showed tumor-specific replication, as visualized by real-time imaging. These findings indicate that neither auxotrophic mutations, nor vaccinia virus deficient for the thymidine kinase gene, nor anaerobic growth conditions were required for tumor specificity and intratumoral replication. We observed localization of tumors by light-emitting microorganisms in immunocompetent and in immunocompromised rodents with syngeneic and allogeneic tumors. Based on their 'tumor-finding' nature, bacteria and viruses may be designed to carry multiple genes for detection and treatment of cancer.

The presence of bacteria and viruses in human tumors has been recognized for more than 50 years[1]. Large numbers of bacteria[2,3] as well as viral particles have been found in tumors excised from patients[4–7].

To demonstrate the survival of bacteria in tumors, spores of the obligate anaerobe *Clostridium pasteurianum* have been injected intravenously into tumor-bearing mice and found to replicate in the hypoxic center of the tumor[8]. Intratumoral and intravenous (i.v.) injection of auxotrophic mutants of *S. typhimurium* results in elevated bacterial titers in the tumor tissues[9]. Engineered retroviral and adenoviral vectors have been administered intratumorally and systemically to tumorous animals, resulting in reduction of tumor size[10,11] and metastatic activity[10,12,13], as well as in decreased angiogenesis[13]. To determine the location of viral particles in rodents with tumors, vaccinia virus carrying the firefly luciferase expression cassette has been injected intravenously[14–17]. Subsequent luciferase assays of homogenates of excised individual organs and tumors reveal a 3- to 500-fold enhancement of light emission in tumor samples, showing the accumulation of viral particles.

To determine the spatial and temporal progression of infections in live animals with implanted tumors, tracing the movement of bacteria or virions is crucial. Isolated structural genes encoding light-emitting proteins, such as luciferases and fluorescent proteins[18–24], allow the detection of bacteria based on luciferase-catalyzed light emission or fluorescence[25–31]. Transfer of the *luxCDABE* gene cluster[32,33] into bacteria results in continuous light emission without the need of exogenous substrate[34]. Injection of light-emitting bacteria allows the visualization and localization of bacteria in live mice[35], even through hard tissues[36]. The *Renilla reniformis* luciferase–*Aequorea victoria* green fluorescent (GFP) fusion protein (RUC-GFP)[37,38] allows real-time monitoring of gene activation in live animals based on luciferase activity and GFP fluorescence[39]. The activity of the *RUC-GFP* expression cassette inserted into vaccinia virus DNA (rVV-*RUC-GFP*) has been imaged in both virus-infected mammalian cell cultures and in virus-infected live animals[40].

In this paper, we describe the monitoring of the movement of light-emitting bacteria and vaccinia virus from the injection site to tumor tissue in live animals. We show that bacteria or vaccinia virus survived and replicated in the tumors for weeks without causing bacteremia or viremia. This was observed in both immunocompromised and immunocompetent animals with allogeneic and syngeneic tumors.

RESULTS

Clearance of bacteria and vaccinia virus from nude mice

To follow the fate of bacteria injected intravenously into the animals, we monitored each animal by low-light imaging at 2-d time intervals ($n > 10$). Injection of an attenuated *S. typhimurium*, an intracellular bacterium, immediately resulted in a disseminated light throughout the body of the animal (Fig. 1a). In contrast, injection of *V. cholerae*, an extracellular bacterium, resulted in light emission localized in the liver

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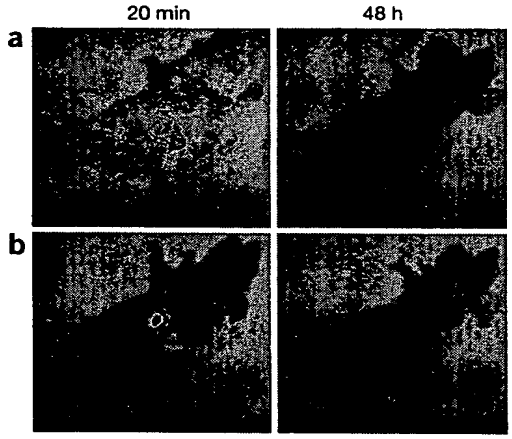

Figure 1 Visualization with the low light imager of the distribution of light-emitting bacteria injected intravenously in nude mice. (a,b) Nude mice were injected with 1×10^8 cells of attenuated *S. typhimurium* (a) and *V. cholerae* (b). Both strains were transformed with pLITE201 carrying the *luxCDABE* operon. Photon collection was for 1 min at 20 min and 48 h after bacterial injection, using the Hamamatsu ARGUS100 imaging system.

region only (Fig. 1b), as did the injection of luminescent *E. coli* (data not shown). Imaging of the same animals 24 and 48 h after injection revealed that all of the detectable light emission from the earlier time points diminished. These findings, together with the complete absence of bacteria in the blood, indicate that the light-emitting bacteria were probably eliminated by the host's immune system. The clearance was independently confirmed by the absence of light emission in all of the excised organs of the same animal (data not shown).

In addition, we examined the distribution of intravenously injected vaccinia virus in nude mice. Nu^-/nu^- mice ($n > 10$) injected with rVV-*RUC-GFP* virus (1×10^8 plaque forming units (pfu)/mouse) were observed once every 3 d over a 2-week period under the low-light imager to detect luciferase-catalyzed light emission in the presence of intravenously injected coelenterazine. In parallel, the animals were also observed under a fluorescence microscope to visualize GFP expression. Two weeks after infection, neither luminescence nor green fluorescence was detected in the live animals with the exception of occasional small skin lesions. However, those minor luminescence and fluorescence signals disappeared within a week as soon as the lesions had healed. One or two weeks after viral injection, the nontumorous animals were killed. At neither time point was luminescence or green fluorescence detected in the excised brain, liver, lung, spleen, kidney or testis (data not shown), indicating that the distribution of rVV-*RUC-GFP* virus via the blood stream did not result in significant infection of healthy organs. This was probably due to prompt clearance by the immune system.

Bacteria and vaccinia virus replicate in nude mouse tumors

To determine the localization of intravenously injected bacteria, we injected nude mice ($n > 20$) with 10-d-old implanted C6 glioma tumors (~500 mm^3) in the right hind leg with 1×10^8 cells of light-emitting *S. typhimurium* or *V. cholerae*. The animals were then monitored each day for 6 d under a low light imager. The initial distribution patterns, determined immediately after injection, were similar to distributions described above (Fig. 2a(i,v)). Two days after injection, however, luminescence diminished in the entire body with the exception of the tumor region (Fig. 2a(ii, vi)). Continued monitoring of the mice at days 4 and 6 after injection showed that after an initial increase, the luminescence started to decrease in the tumors of animals injected with *S. typhimurium* (Fig. 2a(iii–iv)). Remarkably, mice injected with *V. cholerae* exhibited a dramatic increase of light emission in the tumors (Fig. 2a(vii–viii)), indicating an efficient replication of the bacteria (Table 1). The drastically different behavior of *S. typhimurium* and *V. cholerae* in tumors over time may be due to less plasmid (pLITE201) stability in *S. typhimurium*. From the results of bacterial count using homogenized tumor samples, we estimated that one week after bacterial injection, approximately 20% of the *V. cholerae* population retained the plasmid DNA, in comparison to 2% of the *S. typhimurium* population. Furthermore, intravenously injected luminescent *E. coli* DH5α (1×10^8 cells) also replicated rapidly in tumors, similar to *V. cholerae* (data not shown).

In separate experiments, nude mice ($n > 20$) with tumors approximately 500 mm^3 in size were intravenously injected with rVV-*RUC-GFP* virus (1×10^8 pfu/mouse). Mice with tumors approximately 2,500 mm^3 in size were then monitored for GFP fluorescence under a stereomicroscope to determine the site of viral infection and multiplication. GFP expression was first detected in the tumor region 36 h after virus injection. Unexpectedly, an intense green fluorescence was detected in a patch-like pattern exclusively in the tumor region (Fig. 2b(ii)). These patches, often located at the end of blood vessel branches, indicated that the primary site of viral replication was in cells that surround the leaky terminals of capillary vessels. Over time, the GFP signal from the center of these patches started to disappear, and new intense green fluorescent centers appeared in the form of rings at the periphery of the fading patches (data not shown). In addition to GFP, the rVV-*RUC-GFP* virus encoded a functional *R. reniformis* luciferase in the form of a fusion protein. Therefore, immediately after coelenterazine delivery by i.v. injection, a strong luciferase activity was recorded only in the tumor region (Fig. 2c(v)). By lowering the sensitivity of the low light video camera, we detected RUC expression also in localized patches mostly in the periphery of the tumor. These patch-like patterns precisely correlated with the GFP signals (data not shown). Furthermore, we also followed the infection and replication of intravenously injected rVV-*RUC-GFP* virus in the same animal for 20 d after injection (Fig. 2b(iii–v)). A continuously increasing level of GFP fluorescence and luciferase luminescence (Fig. 2c(i–iv)) was indicative of a very efficient viral replication in the tumor tissue. The tumor environment may provide a protective immunoprivileged site for viral replication subsequent to tumor entry and tumor cell

Table 1 Analyses of bacterial and viral titers from homogenized C6 glioma tumors

	Time after i.v. injection of 1×10^8 *V. cholerae*/pLITE201						
	4 h	8 h	16 h	32 h	48 h	Day 4	Day 6
Bacterial titer (cfu/tumor)	$3.79 \times 10^4 \pm 2.93$	$3.14 \times 10^6 \pm 2.45$	$1.08 \times 10^8 \pm 1.3$	$5.97 \times 10^8 \pm 4.26$	$6.84 \times 10^8 \pm 2.2$	$8.82 \times 10^8 \pm 2.68$	$1.06 \times 10^9 \pm 0.48$
	Time after i.v. injection of 1×10^7 pfu rVV-*RUC-GFP* virus						
	36 h		Day 3		Day 5		Day 7
Viral titer (pfu/tumor)	$3.26 \times 10^6 \pm 3.86$		$7.22 \times 10^7 \pm 3.67$		$1.17 \times 10^8 \pm 0.76$		$3.77 \times 10^8 \pm 1.95$

Bacteria or viral particles were injected 13 and 7 d, respectively, after tumor cell implantation. Tumors were excised from animals ($n = 4$). I.V., intravenous. Cfu, colony-forming units. Pfu, plaque-forming units.

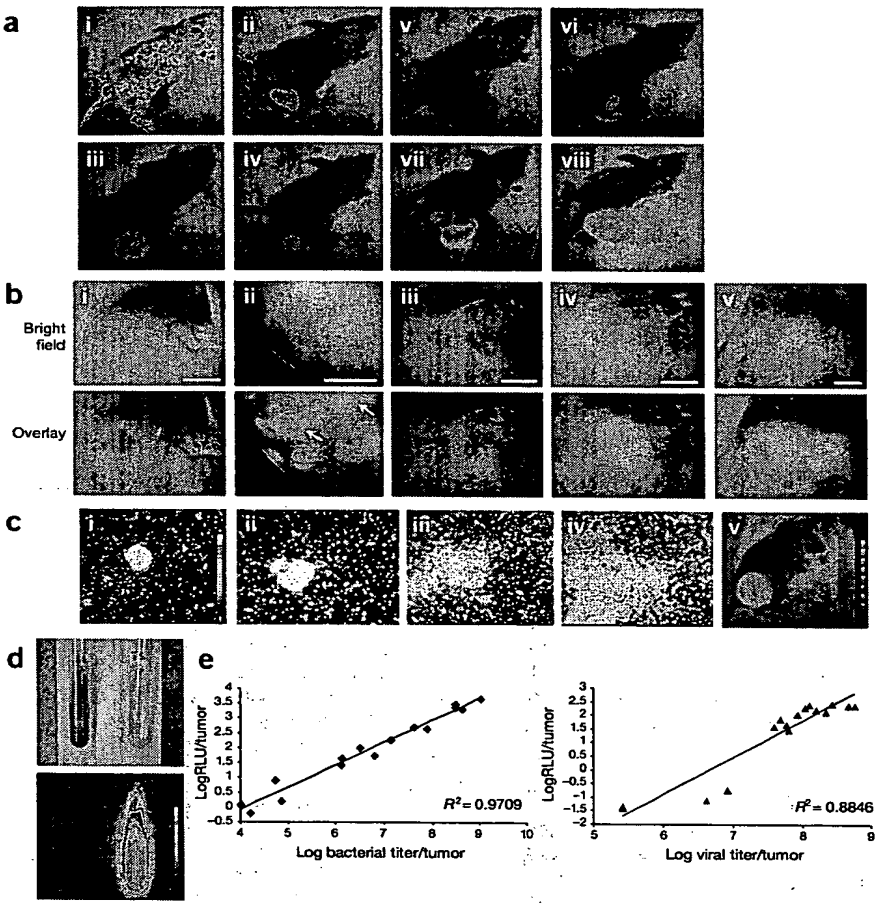

Figure 2 Intravenously injected bacteria and viruses accumulate and replicate in subcutaneous C6 rat glioma tumors in nude mice as visualized by light emission. (**a**) Nude mice with a C6 glioma tumor in the right hind leg were injected with 1×10^8 attenuated *S. typhimurium* (**i–iv**) or attenuated *V. cholerae* (**v–viii**) cells, both transformed with pLITE201 plasmid DNA. Photon collection was carried out for 1 min. Mice injected with *S. typhimurium* exhibited luminescence immediately through the whole animal (**i**). In contrast, luminescence in mice injected with *V. cholerae* was visible shortly thereafter only in the liver area (**v**). Two days after bacterial injection, both groups of mice demonstrated luminescence only in the tumor region (**ii**, **vi**). Light emission in the tumors infected with *S. typhimurium* slowly diminished as seen at 4 (**iii**) and 6 (**iv**) d after bacterial injection. Tumors infected with *V. cholerae* showed a marked increase in light emission 4 (**vii**) and 6 (**viii**) d after injection, suggesting continued replication of the bacteria in the tumor tissues. (**b**) C6 glioma cells (5×10^5) were implanted subcutaneously into the right lateral thigh. At designated days after tumor cell implantation, the animals were injected with 1×10^8 pfu of rVV-*RUC-GFP* virus particles. GFP expression was monitored under a fluorescence stereomicroscope. Bright field (top) and bright field fluorescence overlay (bottom) images of subcutaneous glioma tumor are shown. GFP signal can be observed in tumors as small as 22 mm^3 in size (**i**), or as old as 18 d (~2,500 mm^3 in size) (**ii**). In older tumors, GFP expression was seen in 'patch'-like patterns (indicated by arrows in overlay **ii**). Marker gene expression in the tumor of the same animal can be monitored continuously 4 (**iii**), 7 (**iv**), and 14 (**v**) d after i.v. viral injection. Scale bars = 5 mm. (**c**) Real-time, low-light images of tumors at different time points (36 h (**i**), 3 d (**ii**), 5 d (**iii**) and 7 d (**iv**)) after i.v. injection of 1×10^7 of rVV-*RUC-GFP* indicate the location of RUC-triggered light emission in the presence of i.v. injected coelenterazine (2.5 μg ethanol solution) in anesthetized nude mice. Panel **v** shows the low light and bright field overlay image to indicate the tumor-specific luminescence signal. (**d**) Analysis of the presence of bacteria in blood and tumor fluid samples in overnight culture in Luria Broth medium. Blood and tumor fluid samples were taken from a nude mouse carrying a subcutaneous C6 glioma tumor 1 week after i.v. injection of 1×10^8 light-emitting *V. cholerae*. Fifty microliters of tumor fluid was withdrawn by inserting a 29½ gauge needle directly into the center of the tumor. Only the tumor fluid was shown to be positive for light-emitting bacteria (right tube), whereas no bacteria were detected in the blood (left tube). (**e**) Correlation of luciferase activities with bacterial or viral titers in tumors. Seven or 13 days after C6 glioma cell implantation, nude mice were injected with either 1×10^8 of *V. cholerae*/pLITE201 or with 1×10^7 pfu of rVV-*RUC-GFP*, respectively. At different time points after infection (as shown in **Table 1**), mice were killed. The tumors were excised, homogenized and assayed for bacterial (left) and viral (right) titers. The bacterial luciferase and *Renilla* luciferase activities (reported as relative light units (RLU)) in the tumor homogenates were measured using a luminometer.

infection. The viral replication in the tumor tissues was also independently confirmed by determination of the viral titers in excised tumors (Table 1) and organs obtained at various time points. Homogenates of C6 glioma tumors 1,500 mm^3 in size yielded $2–5 \times 10^8$ plaques in comparison to homogenates of the entire liver or spleen, which yielded less than 2,600 or 100 plaques, respectively, 7 d after injection of 1×10^7 pfu of rVV-*RUC-GFP*. In addition, the luciferase assay using tumor homogenates showed direct correlation between viral titer and luciferase-based light emission (Fig. 2e, right).

The sites of viral infection were determined in exposed tumors, where the GFP fluorescence was found to be concentrated exclusively in the tumor tissue (Fig. 3). Neither the skin nor the nontumorous thigh muscles showed fluorescence. In contrast, cross sections of the tumor revealed strong green fluorescent regions organized as patches in the periphery of the tumor (arrows in Fig. 3b(iii)), indicating the sites where the vaccinia virus–directed gene expression was most active. Analysis of tissue sections under a fluorescence microscope revealed that GFP fluorescence was present in large clusters of cells within the tumor (Fig. 3a). However, no fluorescence was visible in nontumorous tissues.

To determine whether the tumor size and the degree of vascularization affect bacterial entry and colonization, we injected animals with 0-, 2-, 4-, 6-, 8- and 10-d-old subcutaneous glioma tumors with attenuated *S. typhimurium* and *V. cholerae* containing the pLITE201 plasmid DNA. The earliest glioma tumor age at which luminescence was observed was day 8 with a tumor volume of approximately 200 mm^3. When injected intravenously with rVV-*RUC-GFP* virus, nude mice exhibited tumor-specific viral replication 6 d after implantation of

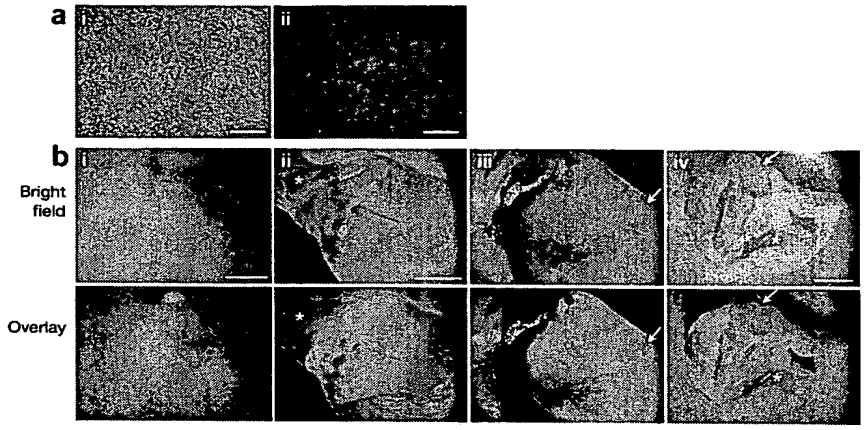

Figure 3 Localization of bacterial colonization and viral infection in subcutaneous C6 glioma tumors. (a) Bright field (i) and fluorescence (ii) images of tumor cells expressing GFP. Frozen sections (30 μm thick) of the glioma tumor tissues were prepared from a nude mouse that has been injected with 1×10^8 pfu of rVV-*RUC-GFP* virus. Scale bars = 50 μm. (b) Five days after the subcutaneous implantation of 5×10^5 C6 glioma cells into the right lateral thigh, 1×10^8 pfu of rVV-*RUC-GFP* virus were injected intravenously. Five days after viral injection, the animal was anesthetized and killed for analysis of GFP expression under fluorescence microscope. The tumor was visualized externally (i), with the overlying skin reflected (ii), in cross section (iii) and in the amputated leg (iv). The strongest GFP expressions are seen as patches located along the outer surface of the tumor on the right (arrows in iii). Sharp difference of GFP expression in tumor tissue and in the normal muscle tissue (arrows in iv) is clearly visible. The reflected skin is marked with asterisks (ii, iv). Scale bars = 5 mm.

C6 glioma cells, as determined by GFP expression 5 d after viral injection in a tumor volume of ~22 mm^3 (Fig. 2b(i)).

Additionally, nude mice bearing subcutaneous PC-3 human prostate tumors were intravenously injected with the attenuated *L. monocytogenes* (1×10^7) transformed with p*SOD-GFP* plasmid DNA. Nearly 27 h after bacterial injection, a weak GFP signal originating from the tumor region was already detectable (Fig. 4a(i)), and no GFP signal was registered elsewhere in the animal. To examine whether the intravenously injected vaccinia virus was able to enter, infect and replicate in tumors other than gliomas, we introduced rVV-*RUC-GFP* into mice with implanted subcutaneous PC-3 human prostate carcinoma. Although the PC-3 implants from which tumors developed grew at a much slower rate than the subcutaneous glioma tumors, these tumors showed the same tumor-specific replication of vaccinia virus (Fig. 4a(ii)). GFP expression was initially detected 3 d after virus injection, and the intense fluorescence lasted throughout the 3-week observation period.

In addition, we also tested the HT1080 human fibrosarcoma cell line, which was stably transformed with a *GFP* expression cassette, and therefore the boundary of the tumors formed by this cell line can be clearly defined on the surface of live animals by fluorescence microscopy. Intravenous injection of *V. cholerae* or *S. typhimurium* (1×10^8) into nude mice carrying such subcutaneous HT1080 fibrosarcoma tumors resulted in tumor-specific colonization by bacteria, and the luminescent signal was seen only within the boundary of the fluorescent tumor (data not shown).

Bacteria and virus replicate in C57 mouse tumors

Immunocompetent C57 mice (n = 2) bearing orthotopic MB-49 murine bladder tumors were injected intravenously with attenuated *V. cholerae*. Bacterial light emission was noted in the bladder region of live animals (Fig. 4b(i)). After abdominal incision, the bladder was exposed, and the light emission was located in the bladder region (Fig. 4b(ii)). Upon surgical removal of the bladder from the mouse, it continued to glow while the rest of the animal body was dark (Fig. 4b(iii–iv)). This experiment showed that even small bladder tumors (<20 mm^3 in size) had the propensity to retain bacteria from the bloodstream in immunocompetent mice. C57 mice with MB-49 tumors were also intravenously injected with rVV-*RUC-GFP*, and 5 d after virus injection, GFP expression was observed only in the bladder tumor region (Fig. 4c).

When Lewis rats with intracranial C6 glioma tumor in the brain were injected intravenously with attenuated *V. cholerae* (1×10^9), low levels of luminescence activity were observed through the skull. Visualization of the excised brain under the imager revealed strong luminescence at the site of the tumor (Fig. 4d(i)). However, control rats injected with phosphate-buffered saline (PBS) alone showed no luminescence. Similarly, weak GFP fluorescence was observed in the surgically exposed intracranial glioma tumors 5 d after i.v. injection of rVV-*RUC-GFP* virus into rats (Fig. 4d(ii–iii)). Taken together, these data show that both bacteria and vaccinia virus did enter and replicate in brain gliomas similarly to the way they replicated in the other tumor models described above.

Bacteria and vaccinia virus reveal the location of metastases

Female nude mice (n = 4) bearing 6-month-old MCF-7 human metastatic mammary carcinoma tumors transformed with pro-insulin-like growth factor (IGF)-II expression cassette[41] (~400–500 mm^3) in the right breast pad were injected intravenously with cells of *V. cholerae*. Two days after injection, the breast tumor was colonized by the bacteria (Fig. 5a(ii)), whereas the liver region became silent (Fig. 5a(i–ii)). Light emission was also visible in the left breast (metastases, ~36 mm^3), indicating the presence of bacteria (Fig. 5a(ii)). Both tumors remained luminescent after 5 d (Fig. 5a(iii)), and strong luminescence activity continued for over 45 d in the primary tumor (data not shown), during which time the mice remained healthy. Experiments using luminescent *E. coli* also showed efficient replication in breast tumors (Fig. 5a(v–vi)).

Female nude mice (n = 4) with established MCF-7 human breast tumors (~400–500 mm^3) were intravenously injected with rVV-*RUC-GFP* virus. A strong GFP expression was observed in the breast tumor region 6 d after virus injection (Fig. 5b(i-ii)). Visualization of cross sections of virus-infected breast tumors revealed fluorescent 'islands' throughout the tumors, with no indication of central or peripheral preference of infection (Fig. 5b(iii)). A smaller metastasized tumor at the left side of the chest also showed intense GFP fluorescence (Fig. 5b (iv–vi)). Metastasized tumor nodules as small as 0.5 mm in diameter on the surface of the excised lung were also positive for GFP fluorescence (Fig. 5b(vii)). The presence of a strong RUC-mediated light emission confirmed the expression of the RUC-GFP fusion protein in these breast tumors, but nowhere else in the body (data not shown). These experiments demonstrate that intravenously delivered VV particles, after entering the tumors and metastases, replicated in the tumor tissue, thereby revealing the location of tumors and metastases.

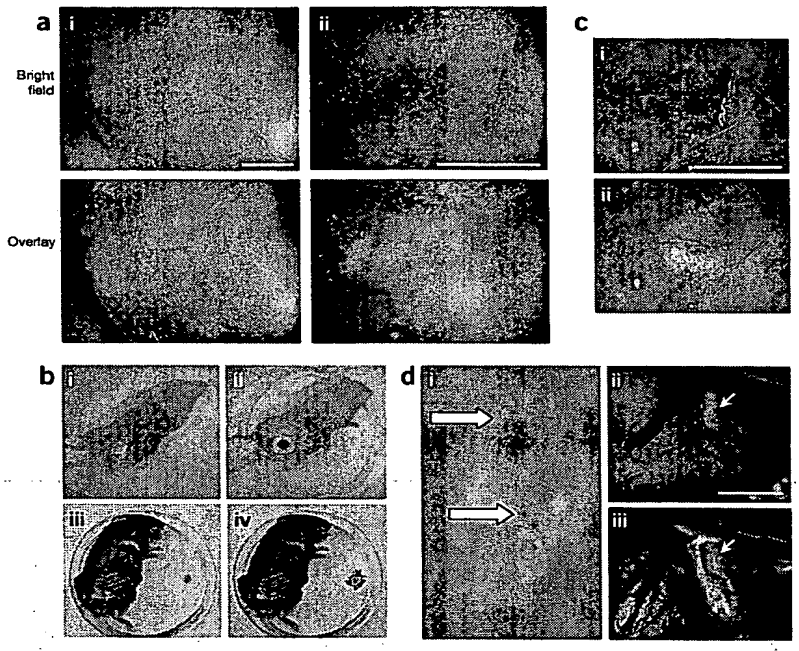

Figure 4 Bacterial and vaccinia virus show tumor-specific localization in different tumorous mice models. (**a**) Intravenously injected *L. monocytogenes* and vaccinia virus accumulate and replicate in subcutaneous PC-3 human prostate tumors in nude mice. (**i**) Mice were injected with 1×10^8 of attenuated *L. monocytogenes* cells transformed with p*SOD-GFP* plasmid DNA carrying the *GFP*-cDNA expression cassette. GFP fluorescence was observed under a fluorescence stereomicroscope. Twenty-seven hours after the injection, GFP signal was detected only in the tumor region. (**ii**) One week after i.v. injection of rVV-*RUC-GFP* at 1×10^8 pfu/mouse, GFP expression was specifically localized to the PC-3 tumors in nude mice. (**b–d**) Bacteria and vaccinia virus show tumor-specific localization in immunocompetent rodents. C57 mice were injected intravenously either with 1×10^8 attenuated *V. cholerae* cells transformed with pLITE201 carrying the *lux* operon (**b**) or with 1×10^8 pfu/mouse of rVV-*RUC-GFP* (**c**). Nine days after delivery of the bacteria, luminescence was noted in the bladder region of the whole animal (**b(i)**). The animal was killed and an abdominal incision was made to expose the bladder. Light emission was limited to the bladder region (**b(ii)**). After removal of the bladder (**b(ii)**) from the mouse, the entire source of light emission was removed (**b(iv)**), as shown by the overlay of the low light photon emission image on the photographic image of the excised bladder. Similarly, 9 d after the delivery of virus, green fluorescence was noted in the surgically exposed bladder tumors (**c(ii)**). (**d**) Lewis rats were injected intravenously either with 1×10^9 attenuated *V. cholerae* cells transformed with pLITE201 (**i**) or with 1×10^8 pfu of rVV-*RUC-GFP* (**ii–iii**). Twenty-four hours after injection of the bacteria, faint luminescence was noted in the head region of the whole animal. The animals were killed. Photon collection from excised tumorous brain was carried out for 1 min, and strong luminescence was confirmed in the tumor region of tumor-bearing brain (**i**, marked with arrows). Similarly, 5 d after viral injection, weak GFP expression was noted in the surgically exposed intracranial tumors in rats (**iii**). Bright field (**c(i)**, **d(ii)**), and bright field/fluorescence overlay (**c(ii)**, **d(iii)**) images are shown. Scale bars = 5 mm.

Do bacteria and vaccinia virus reenter the blood circulation?

To examine whether bacteria are released from tumors, reenter the blood circulation and colonize newly implanted tumors, a second tumor (subcutaneous C6 glioma) was developed on the right hind leg of animals already carrying a strong light-emitting breast tumor. Continuous monitoring of such animals showed no light emission in the second tumor implant, showing that bacteria from the original breast tumor were either not released or were not released in sufficient numbers to be able to colonize the second tumor. However, a repeated i.v. injection of 1×10^8 cells of attenuated *V. cholerae* into the same animal showed strong luminescence activity in the newly implanted tumor concomitantly with continuous light emission from the original tumor. Samples of blood and tumor fluid from subcutaneous C6 glioma tumors colonized by light-emitting *V. cholerae* were collected. Analyses of the blood samples showed complete absence of *V. cholerae*, whereas the tumor samples contained large numbers of luminescent bacteria (Fig. 2d). To determine the level of viral particles released from breast tumors infected with rVV-*RUC-GFP* virus, we implanted C6 glioma cells into the thigh of these mice. No GFP signal was detected in the newly formed glioma tumor, indicating that no viral replication occurred in this tumor. However, both glioma and breast tumors showed strong fluorescence after a repeated i.v. injection of rVV-*RUC-GFP* virus. Based on these findings, we conclude that the release of bacteria and vaccinia virus from infected tumors into blood either did not occur or the released number of microorganisms was insufficient to colonize the newly implanted tumors. The limited release of bacteria or virus from tumors should provide greater safety for bacterium- or virus-mediated detection and therapy of tumors.

DISCUSSION

We show here that bacteria and vaccinia virus gained entry and replicated only in the tumor tissue. Localization of the light-emitting microorganisms in live animals could be followed in real time through low light and fluorescence imaging. Three attenuated bacterial strains, *V. cholerae*, *S. typhimurium* and *L. monocytogenes*, were found to enter and replicate in tumors. Similarly, *E. coli* DH5α also showed tumor-specific localization. In contrast to a previous report[9], no mutations affecting the survival of the bacteria were required for tumor-specific entry and replication of bacteria. We also demonstrated that both primary and metastasized tumors could be visualized using the labeled bacteria. Furthermore, our experiments showed that intravenously injected light-emitting bacteria colonized intracranial tumors in immunocompetent rats and bladder tumors in immunocompetent mice, implying that the tumor microenvironment is an immune privileged site, which provides protection against the host immune system.

Minutes after i.v. injection, the bacteria were distributed throughout the entire animal, and then concentrated in the spleen and the liver as a result of macrophage surveillance. Light emission by bacteria that escaped immune surveillance by entering into the tumor was barely detectable immediately after injection. Owing to rapid bacterial replication, light emission originating from the bacteria within tumors became easily detectable *in vivo*. Visualization experiments using animals carrying breast tumors demonstrated that three sites, namely, the incision wound, the primary tumor and the metastatic tumor were first colonized by the bacteria. One week later, the wound sites became nonluminescent, probably after reconnecting the vasculature with the

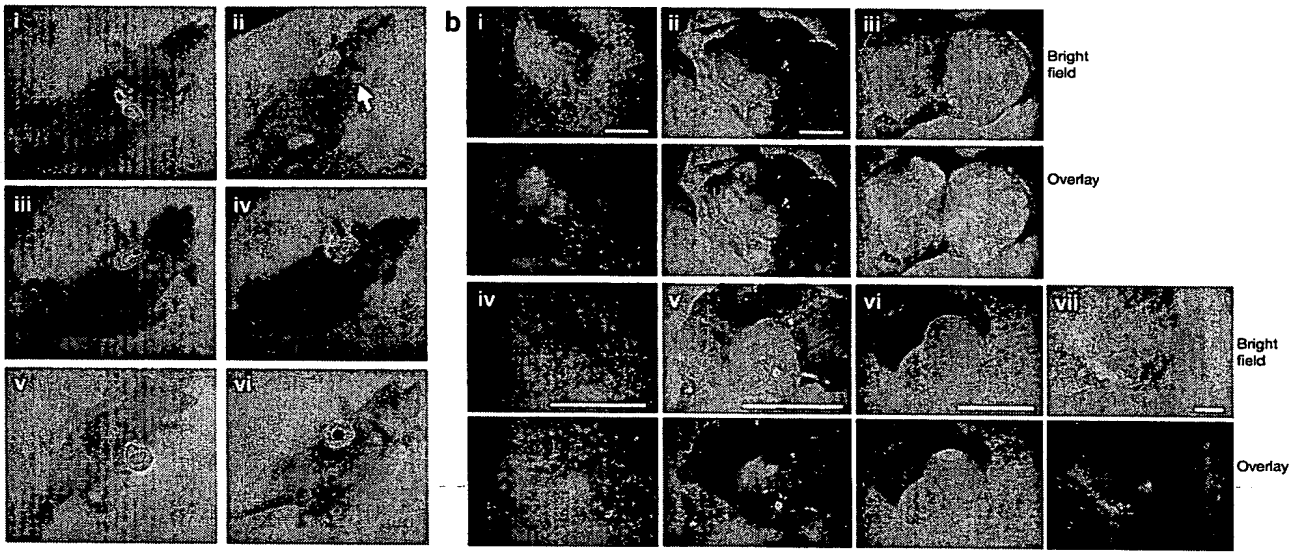

Figure 5 Intravenously delivered light-emitting bacteria and recombinant vaccinia virus mark the location of primary breast tumors and their metastases in nude mice. (a) Nude mice with breast tumors in the right breast pad were injected intravenously with 1×10^8 attenuated *V. cholerae* (i–iv) or with 1×10^8 *E. coli* (v–vi) cells transformed with pLITE201 plasmid DNA. Photon collection was carried out for 1 min. Twenty minutes after bacterial delivery, luminescent *V. cholerae* were observed in the liver (i). Forty-eight hours after injection, light emission was noted in the primary breast tumor located in the right breast area, in a metastatic tumor (arrow) in the left breast area and in the femoral vein incision wound (ii). At 5 d, the light emission was visible only in the tumor regions; there was none in the wound (iii). Eight days after injection of bacteria, the luminescent activity was no longer detectable in the metastatic tumor region but remained strong in the primary breast tumor (iv). Specific localization of *E. coli* for breast tumors in nude mice was also observed 2 d after i.v. injection of bacteria (v, side view; vi, ventral view). (b) Nude mouse carrying breast tumor was injected intravenously with 1×10^8 pfu of rVV-*RUC-GFP* virus. Both the primary tumor (i–iii) and the metastasized tumor (iv–vi) were visualized externally (i,iv), with overlying skin removed (ii,v), and when they were split open (iii,vi). GFP expression in lung metastases in the same animal was also visualized (vii). Scale bars = 5 mm (i–vi), and 1mm (vii).

lymphatic system and restoring the immune surveillance. However, presumably because of an impaired lymphatic system[42], the removal of bacteria from the primary tumor could not occur and therefore continuous light emission was observed over 45 d after injection. This observation showed that only an established tumor possessed the ability to protect bacteria from immune clearance. Therefore, the degree of tumor vascularization and the status of tumor lymphangiogenesis may be the determining factors for tumor colonization by bacteria. The size of different solid tumors did not appear to determine their capability to protect bacteria from the immune system. For example, the minimal size of subcutaneous glioma tumor that was colonized by *V. cholerae* was ~200 mm^3, whereas the bladder tumors (~20 mm^3) in C57 mice were colonized with similar intensity.

Early observations that strains of *Clostridia* preferentially proliferate in necrotic centers of tumors[43] lead to the hypothesis that, unlike normal tissues, the hypoxic environment in tumors provides anaerobic growth conditions[8,44], as it may do for the growth of anaerobic *Bifidobacterium longum*[45]. However, auxotroph mutants of *S. typhimurium* have been shown to multiply in tumors in mice, as found after analysis of homogenized tissues[9,46]. Therefore, anaerobicity in the necrotic center of the tumor alone is not the factor that determines bacterial accumulation in the tumor. In contrast, based on our data, we propose that the entry, survival and replication of bacteria in tumors is dependent on tumor vascularization and tumor immune microenvironment, which provides a sanctuary for a small number of bacteria that will escape clearance by the immune system.

Simultaneously, we also found that intravenously injected vaccinia virus was preferentially localized in different tumors in live animals. Tumor-specific replication by vaccinia virus was demonstrated in subcutaneous C6 glioma and PC-3 tumors, MCF-7 tumors in nude mice, and orthotopic MB-49 tumors in C57 mice. Viral DNA-coded green fluorescence was detected at the site of injection as early as 2 d after injection in contrast to data reported previously[47,48]. Accumulation and replication of vaccinia virus were observed in real time in tumors ranging from 22 to 2,500 mm^3 in volume. Therefore, the unique tumor microenvironment and tumor cell properties, rather than the size of the tumor, seem to determine the entry and survival of viral particles in tumor tissues. These findings are very similar to those found with bacteria. Interestingly, combined i.v. injection of bacteria and vaccinia virus into the same tumor-bearing animal resulted in both bacterial and viral accumulation and replication in the same tumor.

In addition to the visualization of primary tumors, the bacteria and viral particles are also naturally capable of finding small metastatic nodules. Tumor-like nodules as small as 0.5 mm^3 were detected on the surface of the lungs of tumor-bearing mice based on GFP fluorescence after surgical exposure. Since metastatic lesions of small size are difficult to detect, the vaccinia virus–mediated, tumor-specific targeting system may become a clinical tool for sensitive detection and removal of secondary tumors, as well as primary tumors at an early stage.

The results of our experiments demonstrate that the recombinant rVV-*RUC-GFP* of Lister-Institute for Viral Preparations (LIVP) strain replicated specifically in tumorous and in inflamed tissues. Therefore, the tumor-specific infection and replication of vaccinia virus may not be dependent on the availability of metabolites provided by actively dividing cells as suggested by researchers[17], who used a thymidine-

kinase gene–deleted mutant strain of vaccinia virus. Preferential infection of tumor tissues by vaccinia virus and its survival and replication may be the result of fundamental structure and immunological differences between nontumorous and tumorous tissues. Vaccinia virus injected into the bloodstream may enter the tumors through capillaries and replicate in a tumor environment lacking immune protection. In contrast, the rest of circulating viral particles are cleared by the host's immune system shortly after i.v. delivery.

In this study, we demonstrated the real-time visualization of localization, survival and replication of engineered bacteria and vaccinia virus in implanted tumors and their metastases in live animals. We propose that a small number of blood-borne microorganisms may enter tumors through leaky vasculature, thereby escaping the host's immunosurveillance and finding sanctuary in the tumor tissues. These systems may be applied to the detection of tumors and metastases and may allow the development of tumor-specific gene therapy protocols.

METHODS

Bacterial and viral strains. The bacterial strains used were *E. coli* (DH5α), attenuated *S. typhimurium* (SL7207 hisG46, DEL407[aroA544:Tn10]) and attenuated *V. cholerae* (Bengal 2 Serotyp O139, M010 DattRS1). The plasmid pLITE201 (obtained from F. Marincs[49]), containing *luxCDABE*, was used to transform *E. coli*, *S. typhimurium* and *V. cholerae* to produce light-emitting bacterial strains. The plasmid p*SOD-GFP*, carrying the *GFP* gene construct under the control of the *SOD* promoter, was used to transform *L. monocytogenes*.

The viral strain used in this study was LIVP vaccine strain of vaccinia virus. Recombinant vaccinia virus rVV-*RUC-GFP* was constructed by inserting via homologous recombination the *RUC-GFP* cassette[39], which contains the *RUC* and *GFP* cDNA sequences under the control of a synthetic early/late promoter of vaccinia, into the nonessential region of the vaccinia virus genome[40]. The rVV-*RUC-GFP* virus was propagated in CV-1 African green monkey kidney fibroblast cells and purified by centrifugation through a sucrose gradient. The titer of rVV-*RUC-GFP* virus was determined by plaque assay on CV-1 cells and expressed as pfu/ml.

Tumor cell lines. The C6 rat nitrosourea-induced glioma cell line (ATCC) was cultured in RPMI-1640 medium (Cellgro, Mediatech) supplemented with 10% FBS, 100 units/ml penicillin G, 250 ng/ml amphotericin B, and 100 units/ml streptomycin (1×). The PC-3 human prostate carcinoma cell line (ATCC), MB-49 murine bladder carcinoma cells, HT1080 human fibrosarcoma cells transformed with retrovirus carrying the GFP expression cassette (pLEIN, Clontech), and CV-1 cells were cultured in DMEM (Cellgro, Mediatech) supplemented with 10% FBS and 1× penicillin/amphotericin/streptomycin. The MCF-7 human mammary carcinoma cell line (ATCC), permanently transformed with a plasmid carrying pro-IGF-II cDNA (a gift from Daisy deLeon), was cultured in DMEM/F12 medium supplemented with 5% FBS and 560 μg/ml of G418 (Life Technologies).

Recipient animals and tumor models. Five- to six-week-old male BALB/c athymic nu⁻/nu⁻ mice (25–30 g body weight) and Lewis rats (250–300 g) were purchased from Harlan. To generate nude mice carrying subcutaneous glioma tumors, C6 glioma cells were harvested and the number of viable cells was determined by the trypan blue exclusion method. Then 5×10^5 viable cells in 100 μl of PBS were injected subcutaneously into the right lateral thigh of each mouse. Tumor growth was monitored by recording size with a digital caliper. Tumor volume (mm^3) was estimated by the formula $(L \times H \times W)/2$, where L is the length, W is the width and H is the height of the tumor in millimeters. Mice bearing subcutaneous prostate tumors and subcutaneous colon fibrosarcoma were generated over a period of 1 month after subcutaneous implantation of 3×10^6 PC-3 human prostate cells or 5×10^5 retrovirus-transformed HT1080 fibrosarcoma cells, respectively.

Intracerebral glioma tumors were generated by injecting C6 glioma cells into the brain of rats. Rats were anesthetized with sodium pentobarbital (Nembutal sodium solution, Abbot Laboratories; 60 mg/kg body weight). A midline scalp incision (0.5–1 cm) was made, skin was reflected and a 1 mm burr hole was made in the skull located 2 mm to the left and 2.5 mm posterior to the bregma. Tumor cells were pipetted into an insulin syringe fitted with a 29½-gauge needle and mounted in a stereotactic holder. The needle was inserted vertically through the burr hole to a depth of 3 mm. After injection into the brain of a 10 μl volume of either 5×10^5 C6 cells or PBS as control, the needle was kept in place for 15 sec and then withdrawn. The skin incision was closed with surgical clips.

To generate the MCF-7 breast tumor animal model, 4–6-week-old female nude mice were first implanted with 17β-estradiol pellets (0.72 mg per pellet, 90-d release; Innovative Research of America) in the dorsal skin to facilitate breast tumor development and metastasis. One day after estrogen pellet implantation, 1×10^6 MCF-7 human breast carcinoma cells transformed with pro-IGF-II were injected directly into the second left mammary fat pad of each mouse through an incision just below the second nipple. For orthotopic transplants, tumors developed from implanted cells were resected and minced into 1-mm^3 cubes for tissue transplantation into the mammary fat pad. Solid tumor metastases as large as 50 mm^3 may appear in other mammary fat pads 3–6 months after cellular implantation. Extensive lung metastases may also occur within 3–6 months after cellular implantation. C57 mice with an implanted MB-49 murine tumor in the bladder were generated by M. Lilly and kindly provided by I. Fodor.

All animal experiments were carried out in accordance with protocols approved by the Loma Linda University animal research committee and by the Institutional Animal Care and Use Committee (IACUC) of LAB Research International located in San Diego Science Center. Anesthesia of animals was carried out using Nembutal (60 mg/kg body weight). The animals containing recombinant DNA materials and attenuated pathogens were kept in the animal care facilities in Loma Linda University or in LAB Research International at biosafety level two.

I.V. injection of bacteria or vaccinia virus into animals. Unless specified otherwise, 1×10^8 light-emitting bacteria or 1×10^8 pfu of purified rVV-*RUC-GFP* virus, both suspended in 100 μl of PBS, were injected intravenously with a 1-cc insulin syringe equipped with a 29½-gauge needle through the surgically exposed femoral vein. After the injections, the incisions were sutured.

Histology of tumors. Under anesthesia, the animals were killed with Nembutal. The tissues were removed, embedded in Tissue-Tek OCT compound (Miles Scientific) and immediately frozen in liquid nitrogen. Frozen sections (30–60 μm in thickness) were cut using a Reichert-Jung Cryocut 1800 cryostat at –20 °C. GFP fluorescence of the tissues was monitored under a Leica fluorescence microscope and the images were recorded using Photoshop software.

Detection of luminescence and fluorescence. To analyze bacterial luciferase activity, anesthetized animals were placed inside the dark box for photon counting (ARGUS 100 Low Light Imager, Hamamatsu). Photon collection was for 1 min from ventral and dorsal views of the animals, and the images were recorded using Image Pro Plus 3.1 software (Media Cybernetics). A light image was also recorded, which was then superimposed upon the low light image to localize the luminescence activity. RUC activities were determined in anesthetized animals after i.v. injection of a mixture of 5 μl of coelenterazine (0.5 μg/μl diluted ethanol solution) and 95 μl of luciferase assay buffer (0.5 M NaCl; 1 mM EDTA; and 0.1 M potassium phosphate, pH 7.4). Animals were then imaged under the low light imager similarly to imaging animals with bacterial luciferase activities. Luciferase activities in excised and grounded organs were measured using a Turner TD-20e luminometer. Imaging of GFP expression in tumors of live animals was performed using a Leica MZ8 stereo fluorescence microscope equipped with a mercury lamp power supply and a GFP filter (excitation at 470 nm). Images were captured using a SONY DKC-5000 3CCD digital photo camera.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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Light emitting microorganisms and cells for diagnosis and therapy of tumors

Abstract

Described are diagnostic and pharmaceutical compositions comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal, e.g. a luminescent or fluorescent protein. Moreover, the use of said microorganism or cell for tumor-targeting or tumor-imaging is described. For therapeutic uses, said microorganism or cell additionally contain an expressible DNA sequence encoding a protein suitable for tumor therapy, e.g. a cytotoxic or cytostatic protein.

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Claims

1. A diagnostic or pharmaceutical composition comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal.

2. The diagnostic or pharmaceutical composition of claim 1, wherein the protein capable of inducing a detectable signal is a luminescent and/or fluorescent protein.

3. The diagnostic or pharmaceutical composition of claim 1, wherein the protein capable of inducing a detectable signal is a protein inducing a signal detectable by magnetic resonance imaging (MRI) or capable of binding a contrasting agent, chromophore or a ligand required for visualization of tissues.

4. The diagnostic or pharmaceutical composition of any one of claims 1 to 3, wherein the migroorganism is a bacterium or a virus.

5. The diagnostic or pharmaceutical composition of claim 4, wherein the virus is Vaccinia virus.

6. The diagnostic or pharmaceutical composition of claim 4, wherein the bacterium is attenuated Salmonella thyphimurium, attenuated Vibrio cholerae, attenuated Listeria monocytogenes or E. coli.

7. The diagnostic or pharmaceutical composition of claim 1, wherein the cell is a mammalian cell.

8. The diagnostic or pharmaceutical composition of any one of claims 1 to 7, wherein the luminescent or fluorescent protein is luciferase, RFP or GFP.

9. The diagnostic or pharmaceutical composition of any one of claims 1 to 8, wherein the microorganism or cell additionally contains a gene encoding a substrate for a luciferase.

10. Use of the microorganism or cell as defined in any one of claims 1 to 9 for the preparation of a diagnostic composition for tumor-imaging or monitoring a therapeutic tumor treatment.

11. Use according to claim 10, wherein tumor-imaging or monitoring is carried out by MRI.

12. A pharmaceutical composition containing a microorganism or cell as defined in any one of claims 1 to 9, wherein said microorganism or cell furthermore contains one or more expressible DNA sequences encoding (a) protein(s) suitable for tumor therapy and/or elimination of metastatic tumors.

13. The pharmaceutical composition of claim 12, wherein the protein suitable for tumor therapy and/or elimination of metastatic tumors is endostatin or recombinant chimeric toxin PE37/transforming growth factor alpha (TGF-alpha).

14. The pharmaceutical composition according to claim 12 or 13, wherein the expressible DNA sequences are on a BAC, MAC, cyber cell or cyber virus.

15. Use of the microorganism or cell as defined in any one of claims 12 to 14 for the preparation of a pharmaceutical composition for tumor therapy and/or elimination of a metastatic tumor.

16. Use according to claim 15, wherein the tumor is a bladder tumor, breast tumor, prostate tumor, glioma tumor, liver tumor, skin tumor, adenocarcinoma, ovarial carcinoma or pancreatic carcinoma.

Description

[0001] The present invention relates to diagnostic and pharmaceutical compositions comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal, e.g. a luminescent or fluorescent protein.

The present invention also relates to the use of said microorganism or cell for tumor-targeting or tumor-imaging. For therapeutic uses, said microorganism or cell additionally contain an expressible DNA sequence encoding a protein suitable for tumor therapy, e.g. a cytotoxic or cytostatic protein.

[0002] Presence of bacteria in tumors was reported approximately fifty years ago. Several publications substantiated the earlier clinical findings that unexpectedly large numbers of bacteria were discovered in excised tumors from human patients. Investigators argue that chronic infections may predispose cells to malignant growth. Chronic infections of various strains of Chlamydia have been associated with lung and cervical cancer as well as malignant lymphoma. Another well described association between the presence of a specific bacterial species and cancer development is Helicobacter pylori in patients with gastric ulcers. Elevated levels of H. pylori-associated antibodies have been found in patients with duodenal ulcer and gastric adenocarcinoma. These observations demonstrate a concomitant presence of bacteria at tumor sites; however, it was yet not clear whether the microorganisms were the cause of tumor formation or whether the tumorous tissues were more susceptible to bacterial colonization. Intravenously injected strict anaerobic bacteria, Clostridium pasteurianum, into mice replicated selectively in the tumor suggesting a hypoxic microenvironment in the necrotic center. Intravenous injection of attenuated Salmonella typhimurium mutants resulted in elevated bacterial titers in the tumor tissues in comparison to the other organs of mice upon histologic and bacteriologic analyses.

[0003] Similarly, the presence of virus particles was reported in excised human breast tumors as early as 1965. More recently, based on polymerase chain reaction (PCR) data, the human papillomavirus has been claimed to be associated with anogenital tumors and esophageal cancers, breast cancers, and most commonly, cervical cancers. In addition, the presence of hepatitis C virus in human hepatocellular carcinoma, Epstein-Barr virus in squamous cell carcinoma in Kirnura's disease, mouse mammary tumor virus-like paiticles (MMTV) in human breast cancer, SV40 virus in macaque astrocytoma, and herpesvirus in turtle fibropapilloma has also been reported. Surprisingly, the concentration of virus particles in the tumors shows variations among patients. The presence of human papillomavirus in squamous cell carcinomas of the esophagus ranges from 0 to 72% (10-15). In contrast to tumor tissues, no virus particles have been found in tumor-free areas of the esophageal epithelium of the same patient suggesting that the virus particles are located only in the tumor tissues.

[0004] However, so far it could not undoubtedly been shown whether the above discussed microorganisms are responsible for the development of disorders like tumors (except for

papillomaviruses) or whether, e.g., tumors can attract and/or protect viruses or bacteria. Accordingly, there was no basis for the use of such microorganisms for the diagnosis or therapy of tumors. Conventional tumor diagnostic methods, such as MRI (Magnetic Resonance Imaging) and therapeutic methods, e.g. surgery, are invasive and not very sensitive.

[0005] Therefore, it is the object of the present invention to provide a means for the efficient and reliable diagnosis as well as the therapy of tumors which overcomes the disadvantages of the diagnostic and therapeutic approaches presently used.

[0006] According to the present invention this is achieved by the subject matters defined in the claims. When Vaccinia virus (LIVP strain) carrying the light emitting fusion gene construct rVV-ruc-gfp was injected intravenously into nude mice, the virus particles were found to be cleared from all internal organs within 4 days, as determined by extinction of light emission. In contrast, when the fate of the injected Vaccinia virus was similarly followed in nude mice bearing tumors grown from subcutaneously implanted C6 rat glioma cells, virus particles were found to be retained over time in the tumor tissues, resulting in lasting light emission. The presence and amplification of the virus-encoded fusion proteins in the same tumor were monitored in live animals by observing GFP fluorescence under a stereomicroscope and by collecting luciferase-catalyzed light emission under a low-light video-imaging camera. Tumor-specific light emission was detected 4 days after viral injection in nude mice carrying subcutaneous C6 glioma implants ranging in size from 25 to 2500 mm.sup.3. The signal became more intense after the 4th postinjection day and lasted for 30 to 45 days, indicating continued viral replication. Tumor accumulation of rVV-ruc-gfp virus particles was also seen in nude mice carrying subcutaneous tumors developed from implanted PC-3 human prostate cells, and in mice with orthotopically implanted MCF-7 human breast tumors. Further, intracranial C6 rat glioma cell implants in immunocompetent rats and MB-49 human bladder tumor cell implants in C57 mice were also targeted by the Vaccinia virus. Cross sections of a C6 glioma revealed that light emission was clustered in "patches" at the periphery of the tumor where the fast-dividing cells reside. In contrast, cross sections of breast tumors revealed that fluorescent "islands" were distributed throughout the tumors. In addition to primary breast tumors, small metastatic tumors were also detected externally in the contralateral breast region, as well as in nodules on the exposed lung surface, suggesting metastasis to the contralateral breast and lung. In summary, light-emitting cells or microorganims, e.g. Vaccinia virus can be used to detect and treat primary and metastatic tumors.

[0007] Similar results were obtained with light-emitting bacteria (Salmonella, Vibrio, Listeria, E.

coli) which were injected intravenously into mice and which could be visualized in whole animals under a low light imager immediately. No light emission was detected twenty-four hours after bacterial injection in both athymic (nu/nu) mice and immunocompetent C57 mice as a result of clearing by the immune system. In the cutaneous wound of an intravenously injected animal, the bacterial light emission increases and remains detectable up to six days post-injection. In nude mice baring tumors developed from implanted C6 glioma cells, light emission was abolished from the animal entirely twenty-four hours after delivery of bacteria, similar to mice without tumors. However, forty-eight hours post-injection, unexpectedly, a strong, rapidly increasing light emission originated only from the tumor regions was observed. This observation indicates a continuous bacterial replication in the tumor tissue. The extent of light emission is dependent on the bacterial strain used. The homing-in process together with the sustained light emission was also demonstrated in nude mice carrying prostate, bladder, and breast tumors. In addition to primary tumors, metastatic tumors could also be visualized as exemplified in the breast tumor model. Tumor-specific light emission was also observed in immunocompetent C57 mice with bladder tumors as well as in Lewis rats with brain glioma implants. Once in the tumor, the light-emitting bacteria were not observed to be released into the circulation and to re-colonize subsequently implanted tumors in the same animal. Further, mammalian cells expressing the Ruc-GFP fusion protein, upon injection into the bloodstream, were also found to home into and propagate in glioma tumors.

[0008] These findings open the way for (a) designing multifunctional viral vectors useful for the detection of tumors based on signals like light emission and/or for suppression of tumor development and/or angiogenesis signaled by, e.g., light extinction and (b) the development of bacterium- and mammalian cell-based tumor targeting systems in combination with therapeutic gene constructs for the treatment of cancer. These systems have the following advantages: (a) They target the tumor specifically without affecting normal tissue; (b) the expression and secretion of the therapeutic gene constructs are, preferably, under the control of an inducible promoter, enabling secretion to be switched on or off; and (c) the location of the delivery system inside the tumor can be verified by direct visualization before activating gene expression and protein delivery.

[0009] Accordingly, the present invention relates to a diagnostic or pharmaceutical composition comprising a microorganism or cell containing a DNA sequence encoding a detectable protein or a protein capable of inducing a detectable signal.

[0010] Any microorganism or cell is useful for the diagnostic method of the present invention, provided that they replicate in the organism, are not pathogenic for the organism e.g.

attenuated and, are recognized by the immune system of the organism, etc.

[0011] In a preferred embodiment, the diagnostic or pharmaceutical composition comprises a microorganism or cell containing a DNA sequence encoding a luminescent and/or fluorescent protein.

[0012] As used herein, the term "DNA sequence encoding a luminescent and/or fluorescent protein" also comprises a DNA sequence encoding a luminescent and fluorescent protein as fusion protein.

[0013] In an alternative preferred embodiment, the diagnostic or pharmaceutical composition of the present invention comprises a microorganism or cell containing a DNA sequence encoding a protein capable of inducing a signal detectable by magnetic resonance imaging (MRI), e.g. metall binding proteins. Furthermore, the protein can bind contrast agents, chromophores, ligands or compounds required for visualization of tissues.

[0014] Preferably, for transfecting the cells the DNA sequences encoding a luminescent and/or fluorescent protein are present in a vector or an expression vector. A person skilled in the art is familiar with examples thereof. The DNA sequences can also be contained in a recombinant virus containing appropriate expression cassettes. Suitable viruses that may be used in the diagnostic or pharmaceutical composition of the present invention include baculovirus, vaccinia, sindbis virus, Sendai virus, adenovirus, an AAV virus or a parvovirus, such as MVM or H-1. The vector may also be a retrovirus, such as MoMULV, MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For expression in mammals, a suitable promoter is e.g. human cytomegalovirus "immediate early promoter" (pCMV). Furthermore, tissue and/or organ specific promoters are useful. Preferably, the DNA sequences encoding a luminescent and/or fluorescent protein are operatively linked with a promoter allowing high expression. Such promoters, e.g. inducible promoters are well-known to the person skilled in the art.

[0015] For generating the above described DNA sequences and for constructing expression vectors or viruses which contain said DNA sequences, it is possible to use general methods known in the art. These methods include e.g. in vitro recombination techniques, synthetic methods and in vivo recombination methods as described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2.sup.nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., for example. Methods of transfecting cells, of phenotypically selecting transfectants and of expressing the DNA sequences by using the above described vectors are known in the art.

[0016] The person skilled in the art knows DNA sequences encoding luminescent or fluorescent proteins that can be used in the diagnostic or pharmaceutical of the present invention. During the past decade, the identification and isolation of structural genes encoding light-emitting proteins from bacterial luciferase from Vibrio harveyi (Belas et al., Science 218 (1982), 791-793) and from Vibrio fischerii (Foran and Brown, Nucleic acids Res. 16 (1988), 177), firefly luciferase (de Wet et al., Mol. Cell. Biol. 7 (1987), 725-737), aequorin from Aequorea Victoria (Prasher et al., Biochem. 26 (1987), 1326-1332), Renilla luciferase from Renilla reniformis (Lorenz et al., PNAS USA 88 (1991), 4438-4442) and green fluorescent protein from Aequorea victoria (Prasher et al., Gene 111 (1987), 229-233) have been described that allow the tracing of bacteria or viruses based on light emission. Transformation and expression of these genes in bacteria allows detection of bacterial colonies with the aid of the low light imaging camera or individual bacteria under the fluorescent microscope (Engebrecht et al., Science 227 (1985), 1345-1347; Legocki et al., PNAS 83 (1986), 9080-9084; Chalfie et al., Science 263 (1994), 802-805).

[0017] Luciferase genes have been expressed in a variety of organisms. Promoter activation based on light emission, using lux AB fused to the nitrogenase promoter, was demonstrated in Rhizobia residing within the cytoplasm of cells of infected root nodules by low light imaging (Legocki et al., PNAS 83 (1986), 9080-9084; O'Kane et al., J. Plant Mol. Biol. 10 (1988), 387-399). Fusion of the lux A and lux B genes resulted in a fully functional luciferase protein (Escher et al., PNAS 86 (1989), 6528-6532). This fusion gene (Fab2) was introduced into Bacillus subtilis and Bacillus megatherium under the xylose promoter and then fed into insect larvae and was injected into the hemolymph of worms. Imaging of light emission was conducted using a low light video camera. The movement and localization of pathogenic bacteria in transgenic arabidopsis plants, which carry the pathogen-activated PAL promoter-bacterial luciferase fusion gene construct, was demonstrated by localizing Pseudomonas or Ervinia spp. infection under the low light imager as well as in tomato plant and stacks of potatoes (Giacomin and Szalay, Plant Sci. 116 (1996), 59-72).

[0018] All of the luciferases expressed in bacteria require exogenously added substrates such as decanal or coelenterazine for light emission. In contrast, while visualization of GFP fluorescence does not require a substrate, an excitation light source is needed. More recently, the gene cluster encoding the bacterial luciferase and the proteins for providing decanal within the cell, which includes luxCDABE was isolated from Xenorhabdus luminescens (Meighen and Szittner, J. Bacteriol. 174 (1992), 5371-5381) and Photobacterium leiognathi (Lee et al., Eur. J. Biochem. 201 (1991), 161-167) and transferred into bacteria resulting in continuous light

emission independent of exogenously added substrate (Fernandez-Pinas and Wolk, Gene 150 (1994), 169-174). Bacteria containing the complete lux operon sequence, when injected intraperitoneally, intramuscularly, or intravenously, allowed the visualization and localization of bacteria in live mice indicating that the luciferase light emission can penetrate the tissues and can be detected externally (Contag et al., Mol. Microbiol. 18 (1995), 593-603).

[0019] Preferably, the microorganism is a bacterium, e.g. attenuated Particularly preferred is attenuated Salmonella thyphimurium, attenuated Vibrio cholerae or attenuated Listeria monocytogenes or E. coli. Alternatively, viruses such as Vaccinia virus, AAV, a retrovirus etc. are also useful for the diagnostic and therapeutic compositions of the present invention. Preferably, the virus is Vaccinia virus.

[0020] Preferably, the cell of the diagnostic or therapeutic composition of the present invention is a mammalian cell such as stem cells which can be autologous or heterologous concerning the organism.

[0021] In a further preferred embodiment of the diagnostic or therapeutic composition of the present invention the luminescent or fluorescent protein is a luciferase, green fluorescent protein (GFP) or red fluorescent protein (RFP).

[0022] In a particularly preferred embodiment, the microorganism or cell of the diagnostic or pharmaceutical composition of the present invention additionally contains a gene encoding a substrate for the luciferase. In an even more preferred embodiment, the microorganism or cell of the diagnostic or pharmaceutical composition of the present invention contains a ruc-gfp expression cassette which contains the Renilla luciferase (ruc) and Aequorea gfp cDNA sequences under the control of a strong synthetic early/late (PE/L) promoter of Vaccinia or the luxCDABE cassette.

[0023] A preferred use of the microorganisms and cells described above is the preparation of a diagnostic composition for tumor-imaging. The diagnostic composition of the present invention can be used e.g. during surgery, to identify tumors and metastasis. Furthermore, the diagnostic composition of the present invention is useful for monitoring a therapeutic tumor treatment. Suitable devices for analysing the localization or distribution of luminescent and/or fluorescent proteins in an organism, organ or tissue are well known to the person skilled in the art and, furthermore described in the literature cited above as well as the Examples, below. Additionally, the microorganisms and cells can be modified in such a way that they bind metalls and consequently are useful in MRI technology to make this more specific.

[0024] The present invention also relates to a pharmaceutical composition containing a microorganism or cell as described above, wherein said microorganism or cell furthermore contains one or more expressible DNA sequence(s) encoding (a) protein(s) suitable for tumor therapy and/or elimination of metastatic tumors, such as a cytotoxic protein, a cytostatic protein, a protein inhibiting angiogenesis, or a protein stimulating apoptosis. Such proteins are well-known to the person skilled in the art. Furthermore, the protein can be an enzyme converting an inactive substance (pro-drug) administered to the organism into an active substance, i.e. toxin, which is killing the tumor or metastasis. For example, the enzyme can be glucuronidase converting the less toxic form of the chemoterapeutic agent glucuronyldoxorubicin into a more toxic form. Preferably, the gene encoding such an enzyme is directed by a promoter which is inducible additionally ensuring that the conversion of the pro-drug into the toxin only occurs in the target tissue, i.e. tumor. Such promoters are e.g. IPTG-, antibiotic-, heat-, pH-, light-, metall-, aerobic-, host cell-, drug-, cell cycle- or tissue specific-inducible promoters. Additional examples of suitable proteins are human endostatin and the chimeric PE37/TGF-alpha fusion protein. Endostatin is a carboxyterminal peptide of collagen XVIII which has been characterized (Ding et al., PNAS USA 95 (1998), 10443). It has been shown that endostatin inhibits endothelial cell proliferation and migration, induces G1 arrest and apoptosis of endothelial cells in vitro, and has antitumor effect in a variety of tumor models. Intravenous or intramuscular injection of viral DNA and cationic liposome-complexed plasmid DNA encoding endostatin result in limited expression levels of endostatin in tumors. However intratumoral injection of purified endostatin shows remarkable inhibition of tumor growth. Pseudomonas exotoxin is a bacterial toxin secreted by Pseudomonas aeruginosa. PE elicits its cytotoxic effect by inactivating elongation factor 2 (EF-2), which results in blocking of protein synthesis in mammalian cells. Single chain PE is functionally divided into three domains: domain Ia is required for binding to cell surface receptor, domain II is required for translocating the toxin into the target cell cytosol, and domain III is responsible for cytotoxicity by inactivating EF-2. PE40 is derived from wild type Pseudomonas exotoxin that lacks the binding domain Ia. Other proteins such as antibody fragments or protein ligands can be inserted in place of the binding domain. This will render the PE40-ligand fusion protein specific to its receptor. One of the highly specific engineered chimeric toxins is the TGF alpha/PE40 fusion protein, where the C-terminus of TGF alpha polypeptide has been fused in frame with the N-terminus of the PE40 protein. TGF alpha is one of the ligands of epidermal growth factor receptor (EGFR), which has been shown to be preferentially expressed on the surface of a variety of tumor cells. TGF alpha-PE40 fusion protein has been shown to be highly toxic to tumor cells with elevated EGFRs on the cell surface and while it is less toxic to nearby cells displaying fewer numbers of surface EGFR. The toxicity of TGF alpha-PE40 chimeric protein is

dependent on a proteolytic processing step to convert the chimeric protein into its active form, which is carried out by the target. To overcome the requirement for proteolysis, a new chimeric toxin protein that does not require processing has been constructed by Theuer and coworkers (J.Biol.Chem. 267 (1992), 16872). The novel fusion protein is termed PE37/TGF alpha, which exhibited higher toxicity to tumor cells than the TGF alpha-PE40 fusion protein.

[0025] Thus, in a preferred embodiment of the pharmaceutical composition, the protein suitable for tumor therapy is endostatin (for inhibition of tumor growth) or recombinant chimeric toxin PE37/transforming growth factor alpha (TGF-alpha) (for cytotoxicity to tumor cells).

[0026] Moreover, the delivery system of the present application even allows the application of compounds which could so far not be used for tumor therapy due to their high toxicity when systemicly applied. Such compounds include proteins inhibiting elongation factors, proteins binding to ribosomal subunits, proteins modifying nucleotides, nucleases, proteases or cytokines (e.g. IL-2, IL-12 etc.), since experimental data suggest that the local release of cytokines might have a positive effect on the immunosuppressive status of the tumor.

[0027] Furthermore, the microorganism or cell can contain a BAC (Bacterial Artificial Chromosome) or MAC (Mammalian Artificial Chromosome) encoding several or all proteins of a specific pathway, e.g. anti-angionesis, apoptosis, woundhealing-pathway or anti-tumor growth. Additionally the cell can be cyber cell or cyber virus endocing these proteins.

[0028] For administration, the microorganisms or cells of the present invention are preferably combined with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emuslions, various types of wetting agents, sterile solutions etc.. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the microorganisms or cells may be effected by different ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The preferred route of administration is intravenous injection The route of administration, of course, depends on the nature of the tumor and the kind of microorganisms or cells contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physian and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind, size and localization of the tumor, general health and other drugs being adminstered concurrently.

[0029] Preferred tumors that can be treated with the microorganisms or cells of the present invention are bladder tumors, breast tumors, prostate tumors, glioma tumors, adenocarcinomas, ovarial carcinomas, and pancreatic carcinomas; liver tumors, skin tumors.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] FIG. 1: External Imaging of GFP Expression in Subcutaneous C6 Glioma Tumors in Nude Mice

[0031] C6 glioma cells (5.times.10.sup.5) were implanted subcutaneously into the right lateral thigh. At designated days after tumor cell implantation, the animals were infected intravenously with 1.times.10.sup.8 pfu of rVV-ruc-gfp virus particles. GFP expression was monitored under a fluorescence stereomicroscope. Bright field (top), fluorescence (middle), and bright field, fluorescence overlay (bottom) images of subcutaneous glioma tumor are shown. GFP signal can be observed in tumors as small as 22 mm.sup.3 in size (B-B"), or as old as 18 days (about 2500 mm.sup.3 in size) (A-A"). In older tumors, GFP expression was seen in "patch"-like patterns (indicated by arrows in A'). Marker gene expression in the tumor of the same animal can be monitored continuously 4 (C-C"), 7 (D-D"), and 14 (E-E") days after intravenous viral injection. (Bars=5 mm.)

[0032] FIG. 2: Visualization of Tumor Angiogenesis

[0033] C6 glioma cells (5.times.10.sup.5) were implanted subcutaneously into the right lateral thigh of nude mice. Ten days after tumor cell implantation, the animals were infected intravenously with 1.times.10.sup.8 pfu of rVV-ruc-gfp. GFP expression was monitored 7 days post-viral injection. Vascularization at the surface of the subcutaneous C6 glioma tumor is shown against the bright green fluorescent background in the tumor following Vaccinia-mediated gene expressions. Bright field (A), fluorescence (B), and bright field, fluorescence overlay (C) images of subcutaneous glioma tumor are illustrated. (Bars=5 mm.)

[0034] FIG. 3: Expression of GFP in Subcutaneous Glioma Tumor of the Same Animal

[0035] Five days after the subcutaneous implantation of 5.times.10.sup.5 C6 glioma cells into the right lateral thigh, 10.sup.8 of rVV-ruc-gfp virus particles were injected intravenously. Five days after viral injection, the animal was anesthetized and sacrificed for analysis of GFP expression under fluorescence microscope. The tumor was visualized externally (A-K), with

the overlying skin reflected (B-B"), in cross section (C-C"), and in the amputated leg (D-D"). Bright field (A), fluorescence (B), and bright field, fluorescence overlay (C) images of subcutaneous glioma tumor are illustrated. The strongest GFP expressions are seen as patches located along the outer surface of the tumor on the right (double arrows in C-C"). Sharp difference of GFP expression in tumor tissue and in the normal muscle tissue (arrows in D-D") is clearly visible. Asterisks mark the reflected skin (B-B" and D-D"). (Bars=5 mm.)

[0036] FIG. 4: Bright Field (A) and Fluorescence (B) Images of Tumor Cells Expressing GFP

[0037] Frozen sections (30 .mu.m thick) of the glioma tumor tissues were prepared from a nude mouse that has been intravenously injected with 1.times.10.sup.8 of rVV-rucgfp virus particles. (Bars=50 .mu.m.)

[0038] FIG. 5: Low Light Image of the Anesthetized Nude Mouse to Indicate the Location of Renilla luciferase-Triggered Light Emission in the Presence of Intravenously Injected Substrate Coelenterazine (5 .mu.g Ethanol Solution)

[0039] FIG. 6: Monitoring Tumor-Specific Viral Infection Based on GFP Gene Expression in a Variety of Tumor Models

[0040] including subcutaneous PC-3 human prostate tumor (A-A") and MCF-7 human breast tumor (B-B") in nude mice, intracranial C6 rat glioma tumor (C-C", arrows indicate the location of the tumor) in Lewis rats, and MB-49 human bladder tumor (D-D") in C57 mice. Animals were monitored 7 days after intravenous injections of 1.times.10.sup.8 of rVV-ruc-gfp virus particles. Bright field (top), fluorescence (middle), and bright field, fluorescence overlay (bottom) images of the tumor are illustrated. (Bars=5 mm.)

[0041] FIG. 7: Monitoring Vaccinia-Mediated GFP Expression in a Breast Tumor Model

[0042] Nude mouse carrying breast tumor was injected intravenously with 1.times.10.sup.8 of rVV-ruc-gfp virus particles. Both the primary tumor (A-A", B-B", and C-C") and the metastasized tumor (D-D", E-E", and F-F") were visualized externally (A-A" and D-D"), with overlying skin removed (B-B" and E-E"), and when they were split open (C-C" and F-F") in a set of bright field, fluorescence (') and bright field, fluorescence overlay (") images. GFP expression in lung metastases in the same animal was also visualized (G-G"). (Bars=5 mm (A-A" to F-F"), and Bars=1 mm (G-G").

[0043] FIG. 8: Visualization of the Clearance of Light Emitting Bacteria From Nude Mice Based on the Detection of Light Emission Under the Low Light Imager

[0044] Nude mice were intravenously injected with 10.sup.7 cells of attenuated S. typhimurium (A, B) and V. cholera (C, D). Both strains were transformed with pLITE201 carrying the lux operon. Photon collection was done 20 min (A, C) and 2 days (B, D) after bacterial injections.

[0045] FIG. 9: Homing of Glioma Tumors by Attenuated Bacteria

[0046] Nude mice with a C6 glioma tumor in the right hind leg were intravenously injected with 10.sup.7 attenuated S. typhimurium (A-D) and with V. cholera (E-H) both transformed with pLITE201 plasmid DNA encoding the lux operon. Photon collection was carried out for one minute under the low light imager. Mice injected with S. typhimurium exhibited luminescence immediately through the whole animal (A). In contrast, luminescence in the mice injected with V. cholera was visible in the liver area (E). Two days after bacterial injection, both groups of mice demonstrated luminescence only in the tumor region (B, F). The light emission in the tumors infected with S. typhimurium slowly diminished four (C) and six (D) days after bacterial injection. Tumors infected with V. cholera showed enormously increased light emission four (G) and six (H) days after injection suggesting continued replication of the bacteria in the tumor tissues.

[0047] FIG. 10: Homing in of Bacteria onto Breast Tumors

[0048] Nude mice with breast tumors in the right breast pad were intravenously injected with 10.sup.7 attenuated V. cholera (A-D) and with 10.sup.7 E. coli (E-F) transformed with pLITE201 plasmid DNA encoding the lux operon. Photon collection was carried out for one minute under the low light imager. Twenty minutes after bacterial delivery, luminescent V. cholera were observed in the liver (A). Forty-eight hours after injection, light emission was noted in the primary breast tumor in the right breast area and a metastatic tumor (arrow) in the left breast area, and in the incision wound (B). At five days, the light emission was visible only in the tumor regions, and non at the wound (C). Eight days after bacterial injection, the luminescent activity was abolished from the smaller tumor region but remained strong in the primary breast tumor (D). Homing in of E. coli onto breast tumors in nude mice was also observed two days after intravenous bacterial injection (E: side view, F: ventral view).

[0049] FIG. 11: Homing in of Bacteria Onto Bladder Tumors in C57 Mice

[0050] C57 mice were intravenously injected with 10.sup.7 attenuated V. cholera transformed with pLITE201 encoding the lux operon. Nine days after bacterial delivery, luminescence was noted in the bladder region of the whole animal (A). The animal was sacrificed and an abdominal incision was made to expose the bladder. The light emission was limited to the bladder region (B). With the removal of the bladder (C) from the mouse, the entire source of light emission was removed (D) as demonstrated by the overlay of the low light photon emission image over the photographic image of the excised bladder.

[0051] FIG. 12: Homing in of Bacteria onto Brain Glioma Tumors in Lewis Rats

[0052] Lewis rats were intravenously injected with 10.sup.8 cells of attenuated V. cholera transformed with pLITE201 encoding the lux operon. Twenty-four hours after bacterial injection, faint luminescence was noted in the head region of the whole animal during visualization under the low light imager. The animals were sacrificed and their brain removed. Photon collection was carried out for one minute from rats with (A) and without (B) brain tumors. Strong luminescence was confirmed in regions of the brain of the rats with the brain tumor (marked with arrows in A). Luminescence was completely absent in the control brain tissues (B).

[0053] FIG. 13: Transformed Human Fibrosarcoma Cells Home in on Subcutaneous Glioma Tumors in Nude Mice

[0054] Nude mice with human breast tumors were injected intravenously with 5.times.10.sup.5 human fibrosarcoma cells, which were permanently transformed with retrovirus derived from pLEIN. Seven days post-injection, the animals were anesthetized and monitored under a fluorescent stereomicroscope. Fluorescent cells were noted only in the tumor region of the whole mice through the skin (A1-3). Upon exposure of the tumor tissues by reflection of the overlying skin (B1-3), and in cross sections of the tumors (C1-3), fluorescent patches were visible in distinct regions. Close examination of the organs of the mice showed the presence of small clusters of fluorescent cells in the lungs of the animals, demonstrating the affinity of the fibrosarcoma cells for the lungs in addition to the tumorous tissues (D1-3). (Bars=5 mm (A1-C3),=1 mm (D1-D3)).

[0055] FIG. 14: Homing of Attenuated Listeria monocytogenes into Subcutaneous Prostate Tumors

[0056] Nude mice with subcutaneous human PC3 prostate tumor in the right hind leg were

intravenously injected with 10.sup.7 attenuated L. monocytogenes transformed with pSOD-gfp plasmid DNA carrying the gfp cDNA, GFP fluorescence was observed under a fluorescence stereo microscope. Twenty-seven hours after bacterial injection, GFP signal was detected only in the tumor region. The tumor is shown in a set of visible light (a), fluorescent (b), and visible and fluorescent light overlay (C) images. (Bars=5 mm.)

[0057] The present invention is explained by the examples.

EXAMPLE 1

Materials and Methods

[0058] (A) Bacterium strains. The bacterial strains used were attenuated Salmonella typhimurium (SL7207 hisG46, DEL407[aroA544::Tn10]), attenuated Vibrio cholerae (Bengal 2 Serotyp 0139, M010 DattRS1), and attenuated Listeria monocytogenes (D2 mpl, actA, plcB). The bacterial strains were kindly provided by Prof. W. Gobel (University of Wurzburg, Germany).

[0059] (B) Plasmid constructs. The plasmid pLITE201 containing the luxCDABE cassette was obtained from (Voisey and Marincs, Biotech 24, 1998, 56-58). The plasmid pXylA-dual with the operon sequence of gfp-cDNA, lux AB, lux CD, and lux E under the control of the Xylose promoter was kindly provided by Dr. Phil Hill (University of Nottingham, UK).

[0060] (C) Transformation of Bacteria

[0061] The bacteria were transformed by electroporation.

[0062] (D) Tumor Cell lines. The rat C6 nitrosourea-induced glioma cell line (ATCC, Rockille, Md.) was cultured in RPMI-1640 medium (Cellgro, Mediatech, Inc., Herndon, Va.) supplemented with 10% (v/v) FBS and 1.times.penicillin/streptomycin. The human PC3 prostate carcinoma cell line (ATCC, Rockville, Md.) and the Human MB-49 bladder tumor cells and rat 9L glioma cells were maintained in DMEM medium (Cellgro, Mediatech, Inc., Herndon, Va.) supplemented with L-glutamine and 10% (v/v) FBS. HT1080 fibrosarcoma cells (ATCC, Manassas, Va.) were cultured in F12 minimal essential media (Cellgro, Mediatech, Inc., Herndon, Va.) supplemented with 10% FBS and 1.times.penicillin/streptomycin. The MCF-7 human mammary carcinoma cell line (ATCC, Rockville, Md.), permanently transformed with a plasmid carrying pro-IGF-II cDNA (obtained from Dr. Daisy De Leon, Loma Linda Iuniversity,

Loma Linda, Calif.) was cultured in DMEM/F12 medium supplemented with 5% FBS and 560 .mu.g/ml of G418 (Life Technologies, Grand Island, N.Y.).

[0063] (E) Production and propagation of retrovirus to generate a light-emitting stably transformed cell line. PT67 packing cells (Clontech, Palo Alto, Calif.) were cultured in DMEM medium supplemented with 10% (v/v) FBS. At 70% confluence, PT67 cells were transformed with pLEIN (Clontech, Palo Alto, Calif.) using calcium phosphate precipitation method (Profection Mammalian Transfection Systems, Promega, Madison, Wis.) for 12 hours. Fresh medium was replenished at this time. Retroviral supernatant collected from PT67 cells 48 hours post transformation were filtered through a 0.45 .mu.m filter and was added to target HT1080 cells along with polybrene to a final concentration of 4 .mu.g/ml. The medium was replaced after 24 hours and the cells were treated with G418 selection at 400.mu.g/ml and stepwise increased to 1200 .mu.g/ml.

[0064] (F) Recipient animals and tumor models. Five- to six-week-old male BALB/c athymic nu/nu mice (25-30 g in body weight) and Lewis rats (250-300 g in body weight) were purchased from Harlan (Frederick, Md.). C57BL/6J Min/+ mice were obtained from Jackson Laboratories (Bar Harbor, Me.), Min (multiple intestinal neoplasia) is an autosomal dominant trait involving a nonsense mutation in codon 850 of the murine Apc gene, which renders these animals susceptible to spontaneous intestinal adenoma formation. Female BALB/c athymic nu/nu mice baring MCF-7 human breast tumor implants were generated and kindly provided by Dr. Daisy DeLeon and Dr. Tian (Loma Linda University, Loma Linda, Calif.). C57 mice with orthotopically implanted human MB-49 tumor cells in the bladder were generated and kindly provided by Dr. Istvan Fodor (Loma Linda University, Loma Linda, Calif.). All animal experiments were carried out in accordance with protocol approved by the Loma Linda University animal research committee. The animals containing recombinant DNA materials and attenuated pathogens were kept in Loma Linda University animal care facility under biosafety level two.

[0065] (G) Propagation of recombinant vaccinia Virus. Vaccinia virus Lister strain (LIVP) was used as a wild type virus. Recombinant Vaccinia virus rVV-ruc-gfp was constructed by inserting, via homologous recombination, the ruc-gfp-cassette into the Vaccinia virus genome (Wang et al., Proc. Biolumin. Chemilumin. 9, 1996, 419-422). The virus was amplified in CV-1 cells by addition of virus particles at a multiplicity of infection (MOI) of 0. 1 pfu/cell to CV-1 cell monolayers followed by incubation at 37.degree. C. for 1 h with brief agitation every 10 min. At this time, the supernatant fluid with virus particles was removed, and the cell monolayers were washed once with serum free medium. Complete growth medium was then added and the

cells were incubated at 37.degree. C. rVV-ruc-gfp virions propagated in CV-1 cells were purified through a sucrose gradient. A plaque assay was used 72 h after infection to determine the titer of recombinant virus by staining the cells with 50% crystal violet solution in ethanol.

[0066] (H) Generation of mice carrying tumor implants. To obtain tumors in nude mice, C6 glioma cells were grown, harvested and the cell number was determined by the Trypan Blue exclusion method. Disinfectant was applied to the skin surface, then approximately 5.times.10.sup.5 cells were suspended in 100 .mu.l of phosphate buffered saline (PBS) and injected subcutaneously into the right lateral thigh of each mouse. Tumor growth was monitored by recording the size of the tumor with a digital caliper. Tumor volume (mm.sup.3) was estimated by the formula (L.times.H.times.W)/2, where L is the length, W is the width, and H is the height of the tumor in mm.

[0067] Intracerebral glioma tumors were generated by injecting C6 glioma cells into the head of rats. Prior to injection, rats were anesthetized with sodium pentobarbital (Nembutal.RTM. Sodium solution, Abbot Laboratories, North Chicago, Ill.; 60 mg/kg body weight). A midline scalp incision (0.5-1 cm) was made, the skin was retracted, and a 1 mm burr hole was made in the skull at a location 2 mm to the left and 2.5 mm posterior to the brigma. Tumor cells were pipetted into an insulin syringe, which was fitted with a 29-gauge needle and mounted in a stereotactic holder. The needle was inserted vertically through the burr hole to a depth of 3 mm. After injection into the brain of 5.times.10.sup.5 C6 cells in a 10 .mu.l volume, the needle was kept in place for 15 sec and then withdrawn. The skin incision was closed with surgical clips. Mice bearing subcutaneous prostate tumors were generated over a period of one month following subcutaneous implantation of 3.times.10.sup.6 PC3 human prostate cells.

[0068] MB-49 human bladder tumor cells were implanted in the C57 mouse bladder to produce animals with bladder tumors. To generate animals with breast cancer (Tian and DeLeon, submitted for publication), female nude mice were first implanted with 0.72 mg/90 day-release 17.beta.-estradiol pellets (Innovative Research, Rockville, Md.) in the skin to facilitate breast tumor development and metastasis. One day after estrogen pellet implantation, 1.times.10.sup.6 MCF-7 human breast carcinoma cells transformed with pro-IGF-II (Dull et al., Nature 310 (1984), 777-781) were implanted in the mammary fat pad. For orthotopic transplants, tumors developed from implanted cells were resected and minced into 1-mm.sup.3 cubes for tissue transplantation into the mammary fat pad.

[0069] (I) Assay of Renilla luciferase in live animals. Mice were anesthetized with Nembutal (60 mg/kg body weight) before every Renilla luciferase assay. Renilla luciferase activities were

determined after intravenous injection of a mixture of 5 .mu.l of coelenterazine (0.5 .mu.g/.mu.l diluted ethanol solution) and 95 .mu.l of luciferase assay buffer (0.5 M NaCl; 1 mM EDTA; and 0.1 M potassium phosphate, pH 7.4). Whole live animals were then imaged in a dark box using a Hamamatsu low light video camera, and the images were recorded using Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, Md.). The pseudocolored photon emission image was superimposed onto the gray scale image of the animal in order to precisely locate the site of light emission.

[0070] (J) Fluorescence microscopy of live animals. Mice were anesthesized with Nembutal (60 mg/kg body weight) before tumor visualization. External imaging of GFP expression in live animals was performed using a Leica MZ8 stereo fluorescence microscope equipped with a mercury lamp power supply and a GFP filter (excitation at 470 nm). Images were captured using a SONY DKC-5000 3CCD digital photo camera.

[0071] (K) Detection of luminescence and fluorescence. Immediately before imaging, mice and rats were anesthetized with Nembutal.RTM. (60 mg/kg body weight). The animals were placed inside the dark box for photon counting and recording superimposed images (ARGUS 100, Hamamatsu, Hamamatsu, Japan). Photon collection was for one minute from ventral and dorsal views of the animals. A light image was then recorded and the low light image was then superimposed over the light image to record the location of luminescent activity.

[0072] Imaging of GFP expression in tumors of live animals was performed using a Leica MZ8 stereo fluorescence microscope equipped with a mercury lamp power supply and a GFP filter (excitation at 470 run). Images were captured using a SONY DKC-5000 3CCD digital photo camera.

[0073] (L) Histology of tumor tissues. Under anesthesia, the animals were euthanized with an overdose of Nembutal.RTM.. The tissues of interest were removed, embedded in Tissue-Tek OCT compound (Miles Scientific, Naperville, Ill.) and immediately frozen in liquid nitrogen without fixation. Frozen sections were cut at -20.degree. C. using a Reichert-Jung Cryocut 1800 cryostat. GFP fluorescence of the tissues was monitored under a Leica fluorescence microscope and the images were recorded using Photoshop software.

EXAMPLE 2

Results Obtained by Intravenous Injection of Recombinant Vaccinia Virus rVV-ruc-gfp into Mice

(A) Monitoring of Virus-Mediated Marker Gene Expression in Immunodeficient Mice

[0074] Vaccinia virus (1.times.10.sup.8 pfu) carrying the Renilla luciferase--GFP fusion expression cassette (rVV-ruc-gfp) was introduced intravenously into nude mice with no tumors. The animals were observed once every 3 days over a two-week time period under the low-light imager to monitor luciferase catalyzed light emission immediately after intravenous injection of coelenterazine, and under a fluorescence microscope to visualize GFP expression. Neither apparent luminescence nor green fluorescence was detected in the animals when imaged externally, except at certain locations that had small skin lesions. Such luminescence and fluorescence signals disappeared after a few days once the lesions had healed. Animals were sacrificed one week and two weeks after viral infection, and their organs were removed and examined for the presence of luminescence and GFP fluorescence signals. One week after viral injection, no luminescence or green fluorescence could be detected in brain, liver, lung, spleen, kidney or testis. These results indicated that the rVV-ruc-gfp virus did not show organ specificity after injection and that the virus seemed to be cleared from the animal by the immune system soon after systemic delivery via the bloodstream.

(B) Visualization of Vaccinia Virus-Mediated Marker Gene Expression in Glioma Tumors of Live Nude Mice

[0075] The distribution of injected Vaccinia virus in nude mice bearing subcutaneously implanted C6 glioma tumors was examined. Nude mice with tumors approximately 500 mm.sup.3 in size were injected intravenously with 1.times.10.sup.8 pfu of the rVVruc-gfp virus. Seven days after virus injection, the animals were monitored for GFP expression under a fluorescence microscope to determine the presence of viral infection and multiplication in the tumors, which had grown to approximately 2500 mm.sup.3 in size. Surprisingly, green fluorescence was detected only in the tumor regions in live animals. Seven days after viral injection, the GFP fluorescence was very intensely localized in a patch-like pattern restricted to the tumor region (FIG. 1A-A"). These patches, often seen at the end of blood vessel branches, may have indicated local viral infection of tumor cells that surround the leaky terminals of capillary vessels. During real-time observation of the same tumors, the GFP signal from the center of these patches started to disappear, and new green fluorescent centers appeared in the form of rings at the periphery of the fading patches. The new sites of intense GFP fluorescence may have resulted from progression of the viral infection to nearby cells within the tumor during tumor growth and expansion. After careful examination of the mice, with the exception of the tumor region, no detectable green fluorescence was seen elsewhere on the

body surface or in the dissected organs. This experiment clearly showed that a mature solid tumor could be easily localized by the labeled Vaccinia virus, based on light-emission, and it also demonstrated the affinity of virus particles for the tumor tissue.

[0076] To determine whether tumor size and vascularization are decisive factors for viral retention in tumors, nude mice were intravenously injected with 1.times.10.sup.8 rVV-ruc-gfp Vaccinia virus particles one day after subcutaneous C6 cell implantation. Surprisingly, 4 days after viral injection GFP expression was seen in 5-day-old C6 tumors that had a volume of about 25 mm.sup.3 (FIG. 1B-B"). Examination of labeled Vaccinia virus tumor targeting by visualization of GFP expression in implanted tumors younger than 5 days was not feasible in live mice, since sufficient levels of marker gene expression required approximately 4 days to allow detection under a fluorescence microscope.

[0077] The finding that injection of the rVV-ruc-gfp Vaccinia virus into the bloodstream of the host resulted in GFP expression and accumulation in tumors suitable for non-invasive tumor detection allowed us to follow the entry and replication process of this virus in the same animal in real time (FIG. 1 C-C", D-D" and E-E"). A continuously increasing level of GFP fluorescence was observed in the same animal throughout 20 days following viral injection, which was the time scheduled before sacrificing the animals. Such an increase in detectable fluorescence was indicative of a very strong viral replication in the tumor tissue, the latter appearing to function as a protective immunoprivileged environment for viral replication. Interestingly, the location of blood vessels and the neovascularization within the periphery of the enlarging tumor were readily visible and confirmed by external visualization against a bright green fluorescent background (FIG. 1A-A", D-D", E-E" and FIG. 2).

[0078] To determine the location of viral infection within the tumors, the animals were sacrificed and the skin over the tumor was carefully reflected to expose the tumor. In the exposed tumor, GFP fluorescence was found to be concentrated exclusively in the tumor tissue (FIG. 3B-B" and D-D"). The non-tumorous thigh muscles did not show any fluorescence of viral infection, as indicated by arrows in FIG. 3D-D". The skin overlying the tumor was also non-fluorescent (indicated by asterisks in FIG. 3B-B" and D-D"). Cross sections of the tumor, however, revealed that strong green fluorescent regions were mostly found as patches in the periphery of the tumor (double arrows in FIG. 3C-C") where the actively dividing tumor cells are presumably located.

[0079] To further examine the pattern of viral infection in C6 glioma tumors based on GFP expression, the tumor tissues were sectioned for microscopic analysis under the fluorescence

microscope. Comparative analysis of various tissue sections revealed that GFP fluorescence was present in large clusters of cells within the tumor (FIG. 4), but no fluorescence was visible in normal tissues such as the heart, lung, liver, spleen, and kidney.

[0080] In addition to GFP, the recombinant rVV-ruc-gfp virus carried a second marker gene, which encoded the Renilla luciferase in the form of a fusion protein with GFP. Therefore we were able to directly superimpose the site of GFP fluorescence with light emission from Renilla luciferase in the tumors. Immediately after coelenterazine (substrate for Renilla luciferase) was delivered by intravenous injection, a very strong luciferase activity was recorded only in the tumor region under a low light video camera (FIG. 5). By lowering the sensitivity of the low light video camera to avoid saturation of light detection, we were able to identify the Renilla luciferase gene expression in localized patches in the periphery of the tumor. These patch-like patterns precisely correlated with the GFP signals.

(C) Affinity of Vaccinia Virus Delivered to the Bloodstream for Different Tumors Implanted into Animals

[0081] To determine whether the attraction of the Vaccinia virus was limited to glioma tumors or whether this attraction could be observed in other tumors, recombinant Vaccinia virus was recombinantly introduced into mice that carried different types of implanted tumors. One of these tumor models was a nude mouse with implanted subcutaneous PC-3 human prostate carcinoma. Although the PC3 implants from which tumors developed grew at a much slower rate than the implanted subcutaneous glioma tumors, these tumors showed the same dynamics with regards to Vaccinia virus infection when identical titers (1.times.10.sup.8) were injected intravenously (FIG. 6A-A"). Similar to our findings with glioma tumors, GFP expression was initially detected 4 days after virus injection, and the fluorescence lasted throughout the 3-week observation period.

[0082] Female nude mice with established breast tumors were also used for labeled Vaccinia injections. These breast tumors were allowed to grow for 6 months after the animals received implants of MCF-7 human breast carcinoma cells transformed with pro-IGF-11 cDNA. At the time of Vaccinia virus injection, the tumors had reached maximum growth and the tumor volume (about 400-500 mm.sup.3) did not change significantly during the experimental period. Similar to previous experiments, 6 days after intravenous delivery of 1.times.10.sup.8 rVV-ruc-gfp virus particles, strong GFP expression was observed in the breast tumor region (FIG. 6B-B", FIG. 7A-A" and B-B") and nowhere else in the body.

[0083] Examination of cross sections of virus-infected breast tumors revealed luminescent "islands" throughout the tumors without any indication of central or peripheral preference of infection (FIG. 7C-C"). The MCF-7 tumor cells used in these breast tumor models are known to metastasize and in addition to the primary solid tumor, a smaller metastasized tumor found on the left lateral side of the body showed GFP fluorescence (FIG. 7D-D", E-E", and F-F"). Excised lung tissues were also examined for detection of metastases. Metastasized tumors as small as 0.5 mm in diameter on the surface of the lung were positive for GFP fluorescence (FIG. 7G-G"). The presence of a strong Renilla luciferase-mediated light emission confirmed the expression of the luciferase-GFP fusion protein in these breast tumors but nowhere else in the body when the substrate coelenterazine was injected intravenously into the live animals. These experiments showed that intravenously delivered Vaccinia virus particles were selectively attracted to and replicated in primary and metastasized breast tumors in nude mice, likely as a result of the immunocompromised state of the tumor microenvironment.

[0084] To determine whether virus particles could move out of tumors and re-enter the circulation, we injected C6 glioma cells into the thigh of mice to form a second tumor in animals already carrying a breast tumor infected with labeled Vaccinia virus. If the virus particles were released from the tumor to re-enter the circulation in significant numbers they would be able to colonize the newly implanted glioma tumor. Monitoring of these second tumors showed that no GFP signal was visible in the new glioma tumor 7 and 14 days after implantation of the glioma cells. To demonstrate that the newly implanted glioma tumors could be targeted by labeled Vaccinia virus, a second dose of rVV-ruc-gfp virus (1.times.10.sup.8 pfu) was intravenously injected. Five days later, tumor-specific GFP expression was detected in the newly formed glioma tumor in addition to GFP expression seen in the original breast tumor. These findings suggested that the virus particles in infected tumors were either not released back into the circulation at all, or were not released in sufficient numbers to infect and replicate in a second tumor.

[0085] Two additional tumor models, including Lewis rats with intracranial C6 rat glioma tumors and C57 mice with MB-49 human bladder tumors in the bladder, were used for Vaccinia injections. To determine whether tumor-affinity of virus particles is a phenomenon limited to tumors in nude mice with a diminished T-lymphocyte function or whether it is a general protective property of tumors that may be demonstrated also in immunocompetent animals, Lewis rats with intracranial C6 rat glioma tumors and C57 mice with MB-49 human bladder tumors in the bladder were used. A total of 5.times.10.sup.5 C6 glioma cells in a 100 .mu.l volume were stereotactically implanted in the brains of 2 of 4 immunocompetent Lewis rats, and the tumors were allowed to grow for 5 days. The other 2 rats were injected intracranially

with phosphate-buffered saline to serve as controls. On day six, all 4 rats were intravenously injected with rVV-ruc-gfp virus particles via the femoral vein. Five days after virus injection, all 4 animals were sacrificed, and their brains were carefully excised for analysis by fluorescence microscopy. GFP expression was detected in the brains with implanted intracranial tumors (FIG. 6C-C") while no GFP expression was seen in the control brains. In parallel experiments, C57 mice, with or without bladder tumors, were divided into two groups. One group was injected intravenously with rVV-ruc-gfp Vaccinia virus (1.times.10.sup.8 pfu) and the other with saline solution as control. Five days after virus injection, the animals were sacrificed and examined under the fluorescence microscope. GFP expression was observed in the bladder tumor region in C 57 mice but not in control mice (FIG. 6D-D").

[0086] Taken together, these experiments show that Vaccinia virus particles were selectively accumulated and retained in a variety of tumors, probably protected by the tumor microenvironment, and that they were not able to survive in the non-tumorous tissues of immunocompromised as well as immunocompetent animals. The tumor-targeting process by intravenously injected Vaccinia virus carrying the light-emitting dual marker gene demonstrated the ability of the Vaccinia virus system to detect primary and metastatic tumors in live animals.

EXAMPLE 3

Results of Intravenous Injection of Bacterial and Mammalian Light-Emitting Cells into Mice

(A) Visualization of Light Emitting Bacteria Present in Whole Animals After Intravenous Injection

[0087] To determine the fate of intravenously injected luminescent bacteria in the animals, 10.sup.7 bacteria carrying the pLITE201 plasmid in 50 .mu.l were injected into the left femoral vein under anesthesia. Following closure of the incision with sutures, the mice were monitored under the low light imager (ARGUS 100 Camera System, Hamamatsu, Hamamatsu, Japan) in real time and photons were collected for one minute. The imaging was repeated in two-day time intervals to determine the presence of light emission from a given animal. It was found that the distribution pattern of light emission following an intravenous injection of bacteria into mice was characteristic of the bacterial strains used. Injection of the attenuated V. cholera into the bloodstream resulted in light emission localized in the liver immediately. Injection of S. typhimurium, however, was widely disseminated throughout the body of the animal suggesting a difference in the interaction with host cell system (FIG. 8A-8D). Imaging the same animals 24 and 48 hours post-infection showed that all of the detectable light emission from the earlier

time diminished rapidly and was eliminated completely from the injected animal. These findings suggest that light emitting bacteria injected into the bloodstream via the femoral vein are cleared. This process was confirmed by photon emission analysis of excised organs, which were found to lack light emission. Similar data were obtained in immunocompetent mice and rats suggesting that the removal of bacteria from the blood is efficient in both systems.

(B) Bacteria Home in on Glioma Tumors in Nude Mice

[0088] To determine if bacteria preferentially colonize tumorous tissues, nude mice with ten-day-old tumors (about 500 mm.sup.3) in the tight hind leg were injected intravenously via the femoral vein with 10.sup.7 S. typhimurium or 10.sup.7 V. cholera in a 50 .mu.l volume of bacterial suspension. Following injection, the incision wounds were sutured and the animals were monitored for six days under the low light imager. At each observation time point, photons were collected for exactly one minute. In mice injected with S. typhimurium, luminescent bacteria were disseminated throughout the whole body of the animal similar to the findings in the non-tumorous mice (FIG. 9A). Nude mice injected with V. cholera, demonstrated luminescent activity only in the liver region during the early observation period (FIG. 9E). Regardless of the bacterial strain injected, two days after injection, luminescent activity was observed only in the tumor region (FIGS. 9B and 9F). Monitoring of the mice under the low light imager on days four and six post-injection showed decreased amounts of detectable luminescence in the tumors of animals injected with S. typhimurium (FIGS. 9C and 9D. This finding was in marked contrast with the findings in the tumors of mice injected with V. cholera, which demonstrated not only survival but also propagation of the bacteria in the tumor mass with a dramatic increase in light emission (FIGS. 9G and 9H).

[0089] Nude mice bearing subcutaneous human PC3 prostate tumors in the right hind leg were intravenously injected with 10.sup.7 attenuated L. monocytogenes transformed with pS0D-gfp plasmid DNA carrying the gfp cDNA. GFP fluorescence was observed under a fluorescence stereomicroscope. Twenty-seven hours after bacterial injection, GFP signal was detected only in the tumor region (FIG. 10). No GFP signal was observed in the rest of the animal.

(C) Determination of Minimum Size and Age of Glioma Tumors Necessary for Bacterial Infection.

[0090] The purpose of this experiment was to determine whether the size of the tumor has any influence on its ability to be colonized by bacteria. Tumors were induced in the right hind leg of nude mice by subcutaneous injection of glioma cells as described. On days 0, 2, 4, 6, 8, and

10 of tumor induction, attenuated S. typhimurium and V. cholera with the pLITE201 plasmid were injected intravenously through the femoral vein. Presence of luminescent bacteria in the tumor was determined by photon collection for exactly one minute under the low light imager two and four days post-infection. The tumor volume was also determined by measuring the dimensions with a digital caliper. The earliest time-point when luminescent activity was noted in the tumors was on day eight after tumor induction. Corresponding tumor volumes were approximately 200 mm.sup.3.

(D) Bacteria Home in on Breast Tumors of Nude Mice

[0091] In order to determine whether colonization of tumors is limited to glioma cells or whether this is a general phenomenon observed with all tumors, female nude mice baring tumors in the right breast pad were intravenously injected with 10.sup.7 V. cholera in a 50 .mu.l volume of bacteria suspension. The animals were monitored within the first 10 minutes after inoculation under the low light imager for one minute and demonstrated the typical luminescent pattern in the liver region (FIG. 11A). Two days later, while the liver had become clear of luminescent bacteria, the breast tumor was colonized by the labeled V. cholera. In addition to the main tumor, a metastatic tumor in the left breast demonstrated luminescent activity (FIG. 11B). On day five, the animals had cleared the bacteria that colonized the incision wound, however, the tumors remained luminescent (FIG. 1C). FIG. 11D shows the continued colonization and propagation of the bacteria in the main tumor, while the metastatic, smaller tumor had become cleared. Luminescent activity continued for over 45 days in the right breast tumor. Similar experiments were conducted using E. coli to demonstrate that homing in of tumors by bacteria is not strain dependent (FIGS. 11E and 11F).

[0092] To determine whether the bacteria from the tumor enter the blood circulation in significant quantities to colonize other sites, a second tumor (C6 glioma) was induced in these animals in the right hind led. The tumor was allowed to grow for 10 days. No luminescent activity was observed in the glioma tumor demonstrating the absence of a significant bacteria that would cause colonization of this tumor. However, when the animal was rechallenged with 10.sup.7 attenuated V. cholera intravenously, the leg tumor showed strong luminescent activity.

[0093] The findings of these experiments demonstrate that larger tumors retain bacteria more effectively over time. Furthermore, the bacteria within the tumors do not escape into the blood in sufficient quantities to infect susceptible sites such as other tumors.

(E) Bacteria Home in on Bladder Tumors in Immunocompetent Mice

[0094] C57 mice were intravenously injected with 10.sup.7 attenuated V. cholera transformed with pLITE201 encoding the lux operon. On day nine after bacterial delivery, luminescent activity was recorded by photon collection for one minute under the low light imager. Light emission was noted in the bladder region of the whole animal (FIG. 12A). The animals were sacrificed and an abdominal incision was made to expose the bladder. Luminescent activity was positively confirmed to be limited to the bladder (FIG. 12B). Upon removal of the bladder from the mice, luminescent activity was no longer visible anywhere in the animals, however, the excised bladders continued to demonstrate light emission (FIG. 12C). Based on the results of this experiment, bacteria can target tumors in immunocompetent as well as nude mice. Furthermore, the bacteria can also target smaller tumors.

(F) Bacteria Home in on Glioma Tumors in the Brain of Rats

[0095] Lewis rats with glioma tumors in the brain were intravenously injected with 10.sup.8 attenuated V. cholera with the pLITE201 plasmid through the left femoral vein to determine if bacteria can cross the blood-brain barrier and target tumors in immunocompetent animals. The whole animals were monitored for one minute under the low light imager the following day and low levels of luminescent activity was observed through the skull. The rats were sacrificed and the brain tissue was removed in one piece in order to further evaluate the exact location of the luminescent bacteria. Visualization of the excised brain under the imager demonstrated strong luminescent activity in specific regions of the brain (FIG. 13A). Similar imaging of control rats without brains tumors, which were intravenously injected with the labeled bacteria, demonstrated absence of any luminescent activity (FIG. 13B).

(G) Transformed Human Fibrosarcoma Cells Home in on Subcutaneous Glioma Tumors in Nude Mice

[0096] Nude mice with human breast tumors were injected intravenously with 5.times.10.sup.5 human fibrosarcoma cells, which were permanently transformed with retrovirus derived from pLEIN. Seven days post-injection, the animals were anesthetized Nembutal, and monitored under a fluorescent stereomicroscope. Fluorescent cells were noted only in the tumor region of the whole mice through the skin (FIG. 14A1-3). Upon exposure of the tumor tissues by reflection of the overlying skin (FIG. 14B1-3), and in cross sections of the tumors (FIG. 14C1-3), fluorescent patches were visible in distinct regions. Close examination of the organs of the mice showed the presence of small clusters of fluorescent cells in the lungs of the animals,

demonstrating the affinity of the fibrosarcoma cells for the lungs in addition to the tumorous tissue.

EXAMPLE 4

Construction of Bacterial Plasmid Vectors That Carry the Light-Emitting Protein Encoding Expression Cassettes and the Therapeutic Gene Expression Constructs in Cis Configuration

(A) Rationale

[0097] Using the light-emitting expression systems described above, tumors could be imaged based on light emission for up to 45 days in animals. These findings suggest a remarkable plasmid DNA stability in bacteria in the absence of selection. Therefore, by placing the therapeutic gene cassette in cis configuration with the light-emitting protein expression cassette on the same replicon, light emission can be used as an indicator of therapeutic construct presence and stability.

[0098] In contrast to light-emitting proteins, the therapeutic proteins, endostatin and Pseudomonas exotoxin/TGF alpha fusion protein, are required to be-secreted from the bacteria into the medium or into the cytosol of tumor cells for inhibition of tumor growth. To achieve protein secretion from the extracellularly replicating E. coli cells into the tumor, two constructs with different signal sequences can be designed. For secretion of endostatin, the ompF signal sequence can be placed upstream of the coding sequence of endostatin, which facilitates the secretion into the periplasmic space. To release the endostatin into the medium, an additional protein, the PAS protein, needs to be coexpressed with endostatin. PAS has been shown to cause membrane leakiness and the release of secreted proteins into the medium (Tokugawa et al., J.Biotechnol. 37 (1994), 33; Tokugawa et al., J.Biotechnol. 35 (1994), 69). The second construct for the secretion of Pseudomonas exotoxin/TGF alpha fusion protein from E. coli has the OmpA signal sequence upstream of the fusion gene and the release from the periplasmic space into the medium is facilitated by sequences present in domain II of the exotoxin (Chaudhary et al., PNAS 85 (1988), 2939; Kondo et al., J.Biol.Chem. 263 (1988), 9470; Kihara and Pastan, Bioconj.Chem. 5 (1994), 532). To promote secretion of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein from L. monocytogenenes, the signal sequence of listeriolysin (LLO) (Mengaud et al., Infect.Immun. 56 (1988), 766) can be placed upstream of each coding sequence.

[0099] For regulation of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein

expression levels in bacteria, vectors can be generated where the therapeutic protein encoding genes are under the control of the T7 promoter or the P.sub.spac synthetic promoter (Freitag and Jacobs, Infect.Immun. 67 (1999), 1844). Without exogenous induction, the levels of the therapeutic proteins are low in E. coli and in L. monocytogenes. The minimal levels of therapeutic proteins in bacteria provide greater safety following intravenous injection of the engineered bacteria. In the following, six newly constructed plasmid DNAs for constitutive and regulated expression of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein in E. coli and L. monocytogenes are described. All plasmids to be transferred into E. coli will carry the constitutively expressed bacterial lux operon, and all the plasmids to be transferred into L. monocytogenes will carry the constitutively expressed sod-gfp cassette. Plasmids BSPT#1-ESi and BSPT#2-Pti are able to replicate in E. coli only, and plasmids BSPT#3, #4, #5, and #6 replicate in E. coli and L. monocytogenes.

(B) Construction of Plasmid Vectors for Protein Expression and Secretion from E. coli

[0100] The construction of the endostatin secretion vector to be used in E. coli is as follows. The coding sequence of human endostatin (591 bp) will be amplified by PCR from the plasmid pES3 with the introduction of the required restriction sites on both ends, followed by ligation into a pBluescript (Clontech Corp., USA) cloning vector to generate pBlue-ES. The ompF signal sequence (Nagahari et al., EMBO J. 4 (1985), 3589) is amplified with Taq polymerase and inserted upstream in frame with the endostatin sequence to generate pBlue-ompF/ES. The expression cassette driven by the T7 promoter will be excised, and inserted into the pLITE201 vector described in Example 1(B), above, carrying the luxCDABE cassette, to produce the plasmid pLITE-ompF/ES. The sequence encoding the PAS factor (a 76 amino acid polypeptide) will be amplified from the chromosomal DNA of Vibrio alginolyticus (formerly named Achromobacter iophagus) (NCIB 11038) with Taq polymerase using the primers 5'-GGGAAAGACATGAAACGCTTA3-' and 5'-AAACAACGAGTGAATTAGCGCT-3', and inserted into the multiple cloning sites of pCR-Blunt (Clontech Corp., USA) to create the expression cassette under the control of the lac promoter. The resulting plasmid will be named pCR-PAS. The lac promoter linked to the pas gene will be excised from pCR-PAS and inserted into pLITEompF/ES to yield the final plasmid BSPT#1-ESI.

[0101] Plasmid pVC85 (Pastan, see above) contains a T7 promoter, followed by an ompA signal sequence, and a sequence encoding domain II and III of Pseudomonas exotoxin (PE40). The DNA sequence encoding PE40 will be excised with restriction enzymes and replaced with a fragment of PE37/TGF alpha (Pseudomonas exotoxin A 280-613/TGF alpha) obtained from the plasmid CT4 (Pastan, see above) to create the plasmid pVC85-PE37/TGF

alpha. The expression cassette of ompAPE37/TGF alpha linked to the T7 promoter will be excised and inserted into pLITE201 to yield the final plasmid BSPT#2-PTI.

(C) Construction of Plasmid Vectors for Protein Expression and Secretion From L. monocytogenes

[0102] Genes encoding endostatin or PE37/TGF alpha will be inserted downstream of the listeriolysin (LLO) signal sequence in the plasmid pCHHI to generate pCHHI-ES and pCCHI-PE37/TGF alpha. Constitutive expression of the therapeutic proteins will be obtained by linking the above secretion cassettes to the listeriolysin promoter obtained from the pCHHI vector. The SOD-GFP expression cassette, excised from the plasmid pSOD-GFP (Gotz et al. PNAS in press.) will be inserted into pCHHI-ES to generate BSPT#3-ESc, and into pCCHI-PE37/TGF alpha to generate BSPT#4-PTc. For the expression of the therapeutic proteins under the control of an IPTG inducible promoter, the listeriolysin promoter in BSPT#3-ESc and BSPT#4-PTc will be replaced with the P.sub.spac promoter from the plasmid pSPAC (Yansura and Henner, PNAS USA 81 (1984), 439) to generate BSPT#5-ESi and BSPT#6-PTi. P.sub.spac is a hybrid promoter consisting of the Bacillus subtilis bacteriophage SPO-1 promoter and the lac operator. IPTG-induced GFP expression from the P.sub.spac promoter has been documented in L. monocytogenes in the cytosol of mammalian cells.

EXAMPLE 5

Demonstration of the Expression of Luciferase and GFP in Bacteria and Verification of the Secretion of Endostatin and Recombinant Toxin/TGF Alpha Fusion Protein and Their Function in Cell Culture Assays

[0103] To be able to detect the presence of E. coli and L. monocytogenes in tumor tissues in live animals, the levels of the constitutively expressed luciferase and GFP in bacteria need to be adequate. Therefore, after transformation of recipient E. coli or L. monocytogenes with the constructs described in Example 4, the colonies with the highest luciferase light emission or OFP fluorescence will be selected. In addition to characterizing light emission from each selected colony before intravenous injection, the ability of the selected transformants to secret endostatin and Pseudomonas exotoxin/TGF alpha fusion protein into the medium needs to be confirmed. The presence of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein synthesized within E. coli and L. monocytogenes will be determined by extracting these proteins from the cell pellet. The secreted proteins in the medium will be concentrated and analyzed by gel separation and the quantity will be determined by Western blotting. It is

imperative to determine the percentage of the newly synthesized proteins expressed from each plasmid construct in either E. coli or L. monocytogenes that is present in the medium. It is also essential to confirm, in addition to constitutive expression of endostatin and Pseudomonas exotoxin/TGF alpha fusion protein, that expression can be induced in E. coli and in L. monocytogenes upon the addition of IPTG to the bacterial culture medium. For the design of future tumor therapy protocols, the relative amounts of protein secreted by the constitutive expression system needs to be compared to the induced expression levels for a defined time period first in bacterial cultures. It is equally essential to determine that both proteins when synthesized in E. coli and L. monocytogenes are biologically active if generated from the proposed constructs. Both proteins were synthesized previously in E. coli and were shown to be active.

[0104] The results of the experiments described below should confirm whether endostatin is successfully secreted from E. coli using the OmpF signal peptide in combination with PAS pore forming protein expression. These experiments will also show if the PE40/TGF alpha and PE37/TGF alpha fusion proteins are secreted from bacteria using the OmpA signal peptide in combination with domain II of PE. Further, the listeriolysin signal peptide may also facilitate the secretion of endostatin and the chimeric toxin/TGF alpha fusion protein into the medium as well as into the cytosol of infected tumor cells. Using the migration inhibition assay and the protein synthesis inhibition assay, it can be expected to determine that both proteins secreted into the medium are biologically active. The presence and quantities of these proteins may be regulated by replacing the constitutive promoters with promoters that can be induced by IPTG.

[0105] In addition to the secretion system described below, alternative secretion systems such as the E. coli HlyBD-dependent secretion pathway (Schlor et al., Mol.Gen.Genet. 256 (1997), 306), may be useful. Alternative secretion signals from other gram positive bacteria, such as the Bacillus sp. endoxylanase signal peptide (Choi et al., Appl.Microbiol.Biotechnol. 53 (2000), 640; Jeong and Lee, Biotechnol.Bioeng. 67 (2000), 398) can be introduced.

(A) Confirmation of Endostatin and Pseudomonas Exotoxin/TGF Alpha Fusion Protein Secretion from Bacteria into Growth Medium

[0106] E. coli strains (DH5.alpha. and BL21(.lambda.DE3) will be transformed with BSPT#1-ESi and BSPT#2-PTi plasmid DNA. L. monocytogenes strain EGDA2 will be transformed with plasmids BSPT#3-ESc, BSPT#4-PTc, BSPT#5-ESi, and BSPT#6-PTi individually. After plating on appropriate antibiotic-containing plates, individual colonies will be selected from each transformation mixture. These colonies will be screened under a low light imager and

fluorescence microscope for luciferase and GFP expression, respectively. Three colonies with the most intense light emission from each transformation batch will be chosen for further studies. To verify the secretion of endostatin and Pseudomas exotoxin/TGF alpha fusion protein from each selected transformant, the cells will be grown in minimal medium to log phase. After centrifuging down the bacteria, the supernatants will be passed through a 0.45-.mu.m-pore-size filter, and the bacterium-free medium will be used for precipitation of the secreted proteins. The precipitates will be collected by centrifugation. Pellets will be washed, dried, and re-suspended in sample buffer for protein gel separation. Proteins from aliquots corresponding to 10 .mu.l of bacterial culture will be compared to proteins from 200 .mu.l of culture supernatant after separation in a 10% SDS-polyacrylamide gel. Western blot analysis will be performed using polyclonal antibody against endostatin (following the antibody production protocol described by Timpl, Methods Enzymol. 82 (1982), 472) and monoclonal antibody against TGF alpha (oncogene Research Products, Cambridge, Mass., USA). The optimal growth conditions will be established for secretion by sampling the growth medium at different times during growth. A similar method has been used previously to analyze secreted proteins in Salmonella typhimurium culture supernatant (Kaniga et al., J.Bacteriol. 177 (1995), 3965). By use of these methods the amount of secreted proteins in the bacterial culture medium generated by each of the constructs without induction will be established. To estimate the increase in the amount of secreted proteins in the medium, IPTG-dependent promoter activation experiments will be carried out by adding IPTG to the bacterial culture in log phase for 3 to 6 hours, and the secreted proteins will be assayed as above.

(B) Verification of the Biological Activity of Endostatin Secreted by E. coli and L. monocytogenes Using a Migration Inhibition Assay

[0107] It has been shown that endostatin inhibits vascular endothelial growth factor (VEGF)-induced human umbilical vein endothelial cell (HUVEC) migration. Thus, the biological activity of endostatin secreted by bacteria can be tested using the HUVEC migration assay provided by Cascade Biologics, Portland, Oreg. The inhibition of cell migration will be assessed in 48-well chemotaxis chambers (Neuro Probe, Galthersburgs, Md.) (Polverine et al., Methods Enzymol. 198 (1991), 440). Bacterium-free supernatant from each secretion construct will be added to HUVECs for preincubation for 30 min. After incubation, the HUVECs will be placed in the upper chamber. The migration of HUVECs into the lower chamber induced by VEGF.sub.165 (R&D Systems, Minneapolis, Minn.) will be quantified by microscopic analysis. The concentration of functional endostatin in the medium will be directly proportional to the degree of inhibition of HUVEC migration.

(C) Testing the Cytotoxic Activity of Secreted Recombinant PE Toxin Tumor in Tumor Cell Cultures

[0108] The inhibitory activity of the chimeric toxin in mammalian cells will be measured based on inhibition of de novo protein synthesis by inactivating EF-2 (Carroll and Collier, J.Biol.Chem. 262 (1987), 8707). Aliquots of bacterium-free supernatants obtained from the expression of various recombinant PE secretion constructs in E. coli and in L. monocytogenes will be added to the C6 glioma cells or to HCTI 16 colon carcinoma cells. Following treatment with medium, the mammalian cells will be pulsed with [.sup.3H]-leucine, and the incorporation will be determined in the protein fraction. To determine the presence of secreted chimeric toxin proteins in L. monocytogenes-infected mammalian cells, the bacteria will be eliminated from the medium by gentamicin treatment. The mammalian cells containing L. monocytogenes in the cytosol will be lysed, and the released bacteria removed from the lysate by filtration. The mammalian cell lysate containing the secreted chimeric toxins will be assayed in protein synthesis inhibition experiments. The inhibition of [.sup.3H]-leucine incorporation in tumor cell culture will be directly proportional to the amount of the biologically active chimeric toxin protein in the medium and cell lysate.

EXAMPLE 6

Determination of the Entry, Localization and Distribution of Intravenously Injected Bacteria in Tumors of Live Animals

(A) Rationale

[0109] Since only a small number of intravenously injected bacteria escape the immune system by entering the tumor, their immediate localization is not possible due to limited light emission in live animals. Their location can only be verified by sectioning the tumor to identify the early centers of light emission. Looking at sections at a later time point, bacteria can be seen throughout the entire tumor due to rapid replication. To determine whether one or multiple bacteria enter through the same site, red fluorescent protein can be used to label the extracellularly replicating E. coli and green fluorescent protein for the intracellularly replicating L. monocytogenes. By visualizing the distribution of the red and green fluorescence in tissue sections, the entry sites as well as replication and localization of E. coli and L. monocytogenes can be determined individually and simultaneously in the central or peripheral regions of the tumor. It can be expected that the patterns of entry and distribution obtained in implanted tumors mimic those of spontaneous tumors, accordingly, the bacterium-based diagnosis and

protein therapy will become a valid approach.

[0110] With the experiments described in section (B), below, the entry, replication, and distribution of light-emitting bacteria in spontaneous tumors can be compared to the distribution patterns in implanted tumors. Further, double-labeling experiments will allow the operator to precisely locate the extracellularly replicating E. coli and the intracellularly replicating L. monocytogenes in the same tumor sections. Lastly, it can be determined (subsequent to a five-day bacterial colonization) whether bacteria are distributed evenly in the tumors or preferential localization occurs in the periphery of the tumor or in the necrotic center. A possible reduction in bacterial entry into spontaneously occurring tumors due to the immunocompetence of these animals can be overcome by increasing the number of intravenously injected bacteria.

(B) Intravenous Injection of E. coli Expressing Red Fluorescent Protein and L. monocytogenes Expressing Green Fluorescent Protein into Nude Mice and into Rodents with Implanted and Spontaneous Tumors

[0111] E. coli (DH5.alpha.) carrying the DsRed (Matz et al., Nat.Biotech. 17 (1999), 969) expression cassette under the control of a constitutive promoter are used in this experiment. L. monocytogenes EGD strain derivatives with in-frame deletion in each of the virulence genes were individually labeled with the green fluorescent protein cassette driven by the constitutive SOD promoter.

[0112] The localization and intratumoral distribution of bacteria will first be studied in nude mice with implanted C6 glioma or HCT116 colon carcinoma tumors. C6 glioma or HCT116 colon carcinoma cells (5.times.10.sup.5 in 100 .mu.l) will be subcutaneously injected into the right hind leg of the animals. Twelve days after tumor cell injection, the animals will be anesthetized, and the left femoral vein surgically exposed. Light-emitting bacteria (1.times.10.sup.6 cells re-suspended in 50 .mu.l of saline) will be intravenously injected, and the wound incision will be closed with sutures. Tumors will be measured three times a week using a caliper. Tumor volume will be calculated as follows: small diameter.times.large diameter.times.height/2.

[0113] Intracerebral glioma tumors will be generated by injecting C6 glioma cells into the head of Wistar rats. Rats will be anesthetized with Ketamine (70-100 mg/kg body weight) and Xylazine (8-10 mg/kg body weight). A midline scalp incision (0.5-1 cm) will be made, skin will be reflected, and a 1 mm burr hole will be made in the skull located 2 mm to the left and 2.5 mm posterior to the brigma. Tumor cells will be pipetted into an insulin syringe fitted with a 29-

gauge needle and mounted in a stereotactic holder. The needle will be inserted vertically through the burr hole to a depth of 3 mm. After injection into the brain of a 5 .mu.l volume of either 5.times.10.sup.5 C6 cells or PBS as control, the needle will be kept in place for 15 sec and then withdrawn. The skin incision will be closed with surgical clips. Ten days after cell injection, an intracranial glioma will develop which is 5-10 mm in diameter. The same protocols involving intravenous injection of bacteria into animals with tumors will be followed through the reminder of the proposal.

[0114] The localization of bacteria in the tumor, based on GFP or RFP, will also be analyzed using cryosectioned tumor tissues. A reliable morphological and histological preservation, and reproducible GFP or RFP detection may be obtained using frozen sections after a slow tissue freezing protocol (Shariatmadari et al., Biotechniques 30 (2001), 1282). Briefly, tumor tissues will be removed from the sacrificed animals to a Petri dish containing PBS and dissected into the desired size. The samples will be mixed for 2 h in 4% paraformaldehyde (PFA) in PBS at room temperature. They will be washed once with PBS, and embedded in Tissue-Tek at room temperature, and then kept in the dark at 4.degree. C. for 24 h and slowly frozen at -70.degree. C. Before sectioning, the tissue will be kept at -20.degree. C. for 30 min. Then, 10- to 50-.mu.m-thick sections will be cut with a Reichert-Jung Cryocut 1800 cryostat and collected on poly-L-lysine (1%)-treated microscope slides. During sectioning, the material will be kept at room temperature to avoid several freezing and thawing cycles. Finally, the sections will be rinsed in PBS and mounted in PBS and kept in the dark at 4.degree. C.

[0115] To monitor the entry of light emitting E. coli and L. monocytogenes from the blood stream into the tumor, 27 nude mice will be injected with C6 tumor cells, and 27 nude mice with HCT116 colon carcinoma cells. Twelve days after tumor development, 9 animals from the C6 group and 9 from the HCT116 group will receive an intravenous injection of E. coli with the RFP construct. Another 9 animals from each group will receive an intravenous injection of L. monocytogenes transformed with the GFP construct. The third group of 9 animals from each tumor model will receive both E. coli and L. momocytogenes (1.times.10.sup.6 cells of each). Five hours, 25 hours, and 5 days after injection, three animals of each treatment group will be sacrificed, their tumors excised, and processed individually as described in the above cryosectioning protocol. After freezing, each tumor will be cut into two halves. One half of the tumor will be used for preparing thick sections (60-75 .mu.m), which will be analyzed under a fluorescence stereomicroscope to observe the distribution of bacteria in the sections of tumors obtained from each time point of the experiment. The regions of interest will be identified, thin sectioned, prepared, and analyzed with laser scanning cytometry and under the confocal microscope followed by image reconstruction.

[0116] In parallel experiments, animals with spontaneous tumors, as listed in Table 1, will be obtained and used in intravenous injection experiments with E. coli carrying the bacterial lux operon. Two animals of each tumor model will be used, and the luciferase light emission monitored dally under the low light imager. It is expected that the spontaneously occurring tumors can be imaged similarly to the implanted tumors based on bacterial luciferase expression. Two of the spontaneous tumor models, mice with adenocarcinoma of the large intestine and mice with adenocarcinoma of the mammary tissue, will be used for bacterial localization experiments following intravenous injection of E. coli expressing RFP and L. monocytogenes expressing GFP as described above. It can be expected that these experiments will emphasize the significance of the bacterium-based diagnosis and protein therapy system.

1TABLE 1 Spontaneous tumor animal models Animal Strain Tumor species name description Source Reference Mouse 129/Sv- spontaneous Jackson Zhu et al., Madh3.sup.tmlpar adenocarcinoma Laboratorities Cell 94 of large Bar Harbor, (1988), 703 intestine ME Mouse FVB/N- spontaneous Jackson Zhang et al., TgN(UPII- carcinoma of Laboratorities Cancer Res. SV40T) bladder with Bar Harbor, 59 (1999), 29Xrw metastasis to ME 3512 the liver Mouse FVB- spontaneous Jackson Guy et al., neuN adenocarcinoma Laboratorities PNAS USA (N#202) of mammary Bar Harbor, 89 (1992), tissue ME 10578 Rat F344/ spontaneous Charles River Hosokawa et CrCrlBR carcinoma of Laboratories, al., pituitary Wilmington, Toxicol. MA Pathol. 21 (1993), 283

EXAMPLE 7

Verification of Bacterium-Mediated Tumor Targeting and Bacterium-Secreted Protein Therapy in Rodents With Implanted or Spontaneous Tumors

(A) Rationale

[0117] As shown in the previous examples, intravenous injection of light-emitting bacteria results in entry, replication, and accumulation only in the tumor regions in animals. This process can be monitored by imaging of light emission in tumors. Placing the endostatin and chimeric toxin expressing gene cassettes in cis configuration with a light-emitting gene cassette provides an indirect detection system in vivo for their temporal and spacial delivery via bacteria.

[0118] The endostatin and chimeric toxin gene cassettes are linked to signal peptide encoding sequences, which facilitate the secretion of these proteins into the extracellular space in the tumor or into the cytosol of infected tumor cells. Both proteins secreted from bacteria into the extracellular space of the tumor are expected to function similarly to directly injected purified proteins. Both proteins secreted from L. monocytogenes into the cytosol of the infected tumor cells will resemble the viral delivery system reported earlier for endostatin. The bacterial systems can be used as a constitutive secretion system or as an exogenously added IPTG-activatable secretion system in the tumor. By regulating the expression levels of the therapeutic proteins in bacteria that colonize the tumor, the secreted amount of proteins inhibiting tumor growth can be determined. Without the addition of IPTG, the inhibitory protein secretion from the intravenously injected bacteria will be kept at minimum while in blood circulation. This will provide an added safety to the recipient tumorous animals during delivery of bacteria. Using the BSPT system, the onset and duration of the therapy can be controlled by the addition of IPTG. Upon completion of the treatment, the bacterial delivery system can be eliminated by administration of antibiotics, similar to treating a bacterial infection.

(B) Determination of the Effect of Endostatin and Pseudomonas Exotoxin/TGF Alpha Fusion Protein Secreted by E. coli and L. monocytogenes on Tumor Growth in Animals with Implanted Tumors

[0119] The inhibitory effect of endostatin and the cytotoxicity of the chimeric toxin secreted by E. coli and L. monocytogenes in tumors will be determined as follows. Thirty-five nude mice bearing 10-day-old C6 tumors will be injected with bacterial constructs as follows: (a) Five mice with E. coli engineered to secrete endostatin; (b) Five mice with E. coli engineered to secrete chimeric toxin; (c) Five mice with L. monocytogenes engineered to secrete endostatin; (d) Five mice with L. nionocytogeties engineered to secrete chimeric toxin; (e) Five mice with E. coli secreting endostatin and chimeric toxin; (f) control group: five mice injected with E. coli expressing bacterial luciferase alone, and five mice with L. monocytogenes expressing GFP. At the time of bacteria injection, each tumor volume will be determined. Three days after injection, the replication of bacteria in the tumors will be monitored under a low light imager or under a fluorescence stereomicroscope. The light emission and the tumor volume will be measured daily up to 20 days after bacterial injection. Ten days after injection, one animal from each group will be sacrificed and the levels of the secreted proteins present in the tumor tissue will be analyzed using Western blot analysis. These experiments will result in inhibition of tumor growth in endostatin treated animals or a more dramatic tumor regression in animals treated with chimeric toxin proteins. The tumor growth in control animals is not expected to be affected by the bacteria alone.

[0120] In a follow-up experiment, mice with spontaneous adenocarcinoma of mammary tissue (strain FVB-neuN(N#202), Table 1) will be used to study the effect of secreted proteins on tumor growth. An experimental scheme identical to that described for the C6 tumor analysis will be used. At the completion of tumor therapy, the presence of endostatin or chimeric toxin in the tumor tissue will be determined by Western blot analysis. An identical experimental design will be used to assay the effect of IPTG-induction of endostatin and chimeric toxin production in bacteria in C6 tumors as well as in the spontaneously occurring breast tumor mouse model. It is expected that multiple IPTG induction of protein expression in bacteria might be required for successful tumor therapy.

[0121] At any stage of tumor treatment, it may be required to remove the light emitting and therapeutic gene containing bacteria from the animal. To carry out this experiment, mice with 12-day-old C6 tumors will be intravenously injected with E. coli expressing the bacterial luciferase. Three days after injection, antibiotic therapy will be initiated by intraperitoneal administration of gentamicin (5 mg/kg body weight) twice daily, or the newly discovered clinafloxacin (CL960) (Nichterlein et al., Zentralbl.Bakteriol. 286 (1997), 401). This treatment will be performed for 5 days, and the effect of antibiotics on the bacteria will be monitored by imaging light emission from the animals daily.

[0122] By completing the above experiments, it is expected that endostatin and chimeric toxin proteins secreted into the tumors will cause the inhibition of tumor growth and measurable tumor regression. It is anticipated that tumor regression will be achieved in both groups of rodents with implanted tumors and with spontaneously occurring tumors. Experiments with simultaneous application of secreted endostatin and chimeric toxin proteins in tumor treatment may give the most promising results. The removal of the engineered bacteria from the tumor by administration of antibiotics is an added safety measure of the bacterium-secreted protein therapy (BSPT) of the present invention.

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Tumor-Targeted *Salmonella* Expressing Cytosine Deaminase as an Anticancer Agent

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ABSTRACT

The study was designed to evaluate whether TAPET-CD, an attenuated strain of *Salmonella typhimurium* expressing *Escherichia coli* cytosine deaminase (CD), was capable of converting nontoxic 5-fluorocytosine (5-FC) to the active antitumor agent 5-fluorouracil (5-FU). The antitumor effect of TAPET-CD plus 5-FC against subcutaneously implanted colon tumors was also evaluated. TAPET-CD was given to tumor-bearing mice by a single bolus intravenous administration followed with 5-FC by intraperitoneal administration. TAPET-CD accumulated in tumors at levels 1000-fold higher than that in normal tissues and high levels of 5-FU were detected in tumors in mice treated with both TAPET-CD and 5-FC. No 5-FU could be detected in normal tissues. Inhibition of tumor growth was observed in mice treated with either TAPET-CD alone or TAPET-CD in combination with 5-FC (TAPET-CD/5-FC), but not with 5-FC alone. TAPET-CD/5-FC inhibited tumor growth by 88%–96%, compared to TAPET-CD alone, which inhibited tumor growth by 38%–79%. These data suggest that tumor-targeting *Salmonella* could be used to deliver prodrug-converting enzyme selectively to tumors and produced anti-tumor effects when the corresponding prodrug was also given.

OVERVIEW SUMMARY

TAPET-CD, injected intravenously to tumor-bearing mice, accumulates preferentially in tumors compared to normal tissues. TAPET-CD also converts 5-fluorocytosine (5-FC) to agent 5-fluorouracil (5-FU) selectively in tumors in mice treated with TAPET-CD and 5-FC. No 5-FU can be detected in normal tissues. The combination of TAPET-CD and 5-FC effectively suppresses the growth of murine C38 colon carcinoma and two human colon tumor xenografts, LoVo and WiDr. TAPET-CD plus 5-FC is relatively nontoxic in mice and monkeys at doses that produce therapeutic effects. These studies demonstrate the potential use of attenuated *Salmonella* as a tumor-selective protein delivery vector.

INTRODUCTION

THE PRIMARY LIMITATION of cancer therapy is the lack of selectivity of therapeutic agents to cancer cells. Because of this lack of selectivity, anticancer agents elicit toxicity against normal tissues, which ultimately limits the doses that can be given to cancer patients. Many current discovery and development efforts are devoted to finding anticancer agents that selectively target cancer cells to improve the therapeutic index. An alternative approach is to restrict the distribution of drugs to tumors using local-regional administration methods. In order to increase the exposure of drugs to tumors, techniques such as tumor-specific antibodies also have been used to localize cytotoxic agents in tumor cells. An attenuated strain of *Salmonella typhimurium* (VNP20009; Vion Pharmaceuticals, Inc., New Haven, CT) has recently been developed. The attenuation is partly caused by the disruption of the *msbB* gene (Low *et al.*, 1999b), which regulates the addition of a terminal myristoyl group to lipid A. Lipopolysaccharide isolated from *Salmonella* expressing the mutated lipid A show a markedly diminished ability to induce tumor necrosis factor-α (TNF-α). The attenuation is also due to the deletion of the *purI* gene, causing VNP20009 to require an external purine source for survival (Low *et al.*, 1999a).

Vion Pharmaceuticals, Inc., New Haven, CT 06511.

We have previously shown that VNP20009 could be a good vector for tumor-selective delivery of protein-based antitumor agents. VNP20009 is genetically stable, as demonstrated *in vivo* and *in vitro* (Clairmont *et al.*, 2000). Compared to wild-type *Salmonella*, the pathogenicity or toxicity of VNP20009 is reduced or eliminated more than 10,000-fold. Phase I studies in cancer patients (Toso *et al.*, 2002) and studies of several animal species (Lee *et al.*, 2000) demonstrate a good safety profile for VNP20009. According to animal studies (Zheng *et al.*, 2000), VNP20009 preferentially accumulates in tumors (with tumor to normal tissue ratios of 300–25,000:1), and it persists in tumor tissue for more than 4 weeks. VNP20009 exhibits antitumor properties, with tumor size reduction lasting for up to 50–60 days after a single intravenous administration in a variety of murine models (Luo *et al.*, 2002). Because VNP2009 is sensitive to a number of antibiotics, it can be conveniently eliminated from the body. In contrast, other vector systems such as viruses or liposomes cannot be eliminated easily from the body. In addition, VNP20009 is environmentally friendly because it has difficulty surviving freely in the environment.

Using cytosine deaminase (CD) and green fluorescent protein genes as markers, we further demonstrated that VNP20009 could be used as a tumor-selective vector for delivering antitumor agents (Zheng *et al.*, 2000). TAPET-CD is generated by the incorporation of the CD gene from *Escherichia coli* into VNP20009. CD is an enzyme found in bacteria and fungi, but not in mammalian cells. The enzyme converts 5-fluorocytosine (5-FC), a relatively nontoxic agent, to the cytotoxic antimetabolite 5-fluorouridine (5-FU) (Deonarain *et al.*, 1995). The activated agent is then converted to 5′fluorouridine-5′triphosphate and 5-fluoro-2′deoxyuridine-5′ monophosphate, resulting in the disruption of RNA and DNA synthesis with subsequent toxicity to both quiescent and proliferating cells. 5-FU is currently used in the clinical treatment of colorectal, stomach, head and neck, and breast carcinomas. Although 5-FU is not as toxic as some antitumor alkylating agents, sufficiently high levels of 5-FU have been achieved *in vivo* that are capable of eradicating tumors after retroviral transduction of the CD gene, even when CD incorporates into only 2% of tumor cells (Huber *et al.*, 1994). Cumulative clinical data suggest that the antitumor activity of 5-FU may be directly related to the duration of exposure and its concentration within the tumor. One approach for achieving high and prolonged tumor concentrations of 5-FU involves coadministration of TAPET-CD and 5-FC. TAPET-CD maintains its VNP20009 properties (Lee *et al.*, 2001) and selectively produces high levels of CD in tumors compared to normal tissues (Zheng *et al.*, 2000). We report here that TAPET-CD/5-FC induces prolonged, high concentrations of 5-FU in tumor tissues, causing a reduction in tumor size greater than that induced by TAPET-CD or 5-FC alone.

MATERIALS AND METHODS

TAPET-CD genetic manipulation

The CD gene was isolated from *E. coli* using procedures described by Laliberte and Momparler (1994) CD was initially cloned into pTrxFus (In Vitrogen, Carlsbad, CA) to encode a thioredoxin-CD fusion protein, then the fusion protein gene was subcloned into pTrc99A. The cloned DNA was transformed into *Salmonella* as described (Pawelek *et al.*, 1997).

Cell culture

Murine B16-F10 melanoma cells were obtained from Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX). C38 colon carcinoma cells were obtained from NCI (Frederick, MD). Widr and LoVo colon carcinoma xenografts were obtained from the American Type Culture Collection (Rockville, MD). Cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. At approximately 80% confluence, cells were detached from the flasks by addition of 2 ml of trypsin, resuspended in 25 ml phosphate-buffered saline (PBS), and transferred into a 50-ml Falcon conical centrifuge tube. Cells were pelleted by centrifugation at 4°C for 5 min at 800 revolutions per minute (rpm) in a Beckman (Fullerton, CA) GS-6R refrigerated centrifuge. The supernatant was discarded, and the cell pellet was resuspended in PBS. The tumor cell suspension was kept on ice until implantation into mice.

Animals

Female C57BL/6 and *nu/nu* CD-1 female mice were obtained from Charles River Laboratories (Wilmington, MA). Animals used in the studies were as uniform in age and weight as possible. They were approximately 8 weeks of age, and body weights of C57BL/6 mice and *nu/nu* CD-1 mice ranged from 19–21 g and 23–26 g, respectively. All animals were kept in a well-ventilated room in which a 12-hr light/12-hr dark photoperiod was maintained. Room temperature was maintained between 72°F ± 2°F.

Quantitation of TAPET-CD accumulation and 5FC/5FC conversion in tissues

Animals were inoculated intravenously with 1×10^6 colony-forming units (cfu) of TAPET-CD on day 14 after B16-F10 melanoma tumor implantation. Mice received 300 mg/kg of 5-FC intraperitoneally on day 17, 3 days after TAPET-CD injection. They were killed by inhalation of CO_2 at 10, 30, 90, 270, and 360 min after 5-FC injection. Tissues, including tumor, liver, spleen, brain, whole blood, and bone marrow were weighed and homogenized in PBS, and bacteria were quantitated by plating serial dilutions of the homogenates onto msbB plates, incubating overnight at 37°C, and counting bacterial colonies. The conversion of 5-FC to 5-FU in tissues including tumor, liver, spleen, brain, serum, and bone marrow, was determined by high-performance liquid chromatography (HPLC) analysis. The detection limit for 5-FC and 5-FU was 0.5 μg/g and 0.2 μg/g, respectively.

Antitumor activity of TAPET-CD

For the murine tumor model, C38 tumor tissue was aseptically dissected from a tumor-bearing C57BL/6 mouse. The tumor was mechanically minced into 3–5 mm^3 pieces, and transplanted subcutaneously with a 16-gauge trocar needle into the right flank of C57BL/6 mice under methoxyflurane anesthesia. Fifteen days after transplantation, when C38 tumors had grown to a volume of approximately 300 mm^3, the mice were ran-

domized and divided into 4 groups of 10 animals each. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 were injected in the tail vein with TAPET-CD at 1×10^6 cfu per mouse on day 15. Groups 3 and 4 received daily three intraperitoneal injections of 5-FC at a dose of 300 mg/kg on days 19 through 40.

WiDr cells were implanted subcutaneously (5×10^6 cells) into *nu/nu* CD-1 mice (day 0). By day 21, mice had similar tumor sizes of approximately 250 mm^3 as determined by electronic caliper measurements. They were randomly assigned into 4 groups of 7–8 animals each. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 were injected in the tail vein with TAPET-CD at 1×10^6 cfu per mouse. Groups 3 and 4 received daily three intraperitoneal injections of 5-FC at a dose of 300 mg/kg on days 23 through 44.

LoVo human colon carcinoma cells were implanted subcutaneously into *nu/nu* CD-1 mice. By day 20, tumors had grown to approximately 150 mm^3. Groups 1 and 3 received PBS (0.1 ml). Groups 2 and 4 received a single dose of TAPET-CD at 1×10^6 cfu per mouse through the tail vein. Groups 3 and 4 received daily three intraperitoneal injections of 300 mg/kg 5-FC on days 26 through day 53.

In another control experiment, CD-1 nude mice were implanted with Widr human colon tumors and treated with 5-FU, either alone or in combination with TAPET-CD when tumor reached approximately 250 mm^3. 5-FU was given intraperitoneally to mice 5 days after TAPET-CD administration, once daily for 5 days at 50 mg/kg per day.

Tumor volume was measured in three dimensions twice weekly and calculated with the formula $L \times H \times W/2$, where L, H, and W represent length, height, and width, respectively. Tumor volume was presented as mean ± standard deviation and Student's *t* test was performed for statistical analysis.

RESULTS

TAPET-CD tissue distribution

The distribution of TAPET-CD was determined by quantitating the amount of TAPET-CD in tumors and normal tissues. Three days after TAPET-CD injection, the highest levels of TAPET-CD occurred in tumors ($1.1 \times 10^9 \pm 1.4 \times 10^8$ cfu/g), followed by spleen ($9.3 \times 10^6 \pm 3.1 \times 10^5$ cfu/g) and liver ($2.2 \times 10^5 \pm 8.0 \times 10^4$ cfu/g). The amount of TAPET-CD in brain, bone marrow, and whole blood was approximately four to five orders of magnitude lower than that found in tumors (Fig. 1). These results indicate that TAPET-CD preferentially accumulates and proliferates in tumors rather than in normal tissues. Tissue distribution of TAPET-CD in non-tumor–bearing mice was similar except that approximately 10 times less bacteria were detected in the brain (data not shown).

In Vivo *5-FC/5-FU conversion in tumors by TAPET-CD*

The concentrations of 5-FC in serum and different tissues are shown in Figure 2A. The concentration of 5-FC in the untreated control group was undetectable (data not shown). Similarly, 5-FC could not be detected in mice treated with 60 mg/kg 5-FU. In mice treated with 5-FC only, the average concentration of 5-FC was 1675 μg/ml in serum, 260 μg/g in liver, 119 μg/g in tumor, 103 μg/g in spleen, 57 μg/g in bone marrow, and 13 μg/g in brain at 30 min after administration of 300 mg/kg 5-FC. In mice previously treated with TAPET-CD, the tissue distribution of 5-FC in the above tissues was similar to those of mice treated with 5-FC alone, indicating that TAPET-CD treatment may not change the distribution pattern of 5-FC in the body. The concentrations of 5-FU in serum and different tissues in mice receiving 5-FC, with and without TAPET-CD, were also determined. As shown in Figure 2B, no 5-FU was detected in any tissues of the group treated with 5-FC alone, indicating that no endogenous conversion of 5-FC to 5-FU occurs in the body. In the group treated with 5-FU alone, mice received a single therapeutic dose of 60 mg/kg. The concentrations of 5-FU in the evaluated tissues ranged from 0.7 to 10 μg/g at 30 min after 5-FU injection. The average level of 5-FU in tumors (4 μg/g) was significantly lower than that in serum (10 μg/ml) and liver (6 μg/g) and similar to that in bone marrow (5 μg/g) and spleen (4 μg/g). However, the highest levels of 5-FU were detected in tumor tissues of mice receiving both TAPET-CD and a single dose of 300 mg/kg of 5-FC. The 5-FU levels reached 19 μg/g in tumors 30 min after 5-FC administration, 4.75-fold more than that found in tumors of mice treated with 60 mg/kg of 5-FU. No 5-FU was detected in other tissues in mice receiving TAPET-CD/5-FC. These results indicate that 5-FC was efficiently converted to 5-FU in tumors by CD, which was expressed by high levels of accumulated TAPET-CD. Because TAPET-CD in other tissues was about 1000-fold lower than in tumors, there was insufficient CD in these tissues to convert 5-FC to 5-FU.

Pharmacokinetics of 5-FC and converted 5-FU in tissues

The pharmacokinetics of 5-FC conversion to 5-FU in tumors are shown in Figures 3A and 3B. After a single intraperitoneal injection of 5-FC (300 mg/kg), 5-FC in tumors quickly reached a level of 160 μg/g at 10 min, then gradually declined and became undetectable at 90 min. The comparative 5-FC levels in the tested tissues were: Serum > liver > tumor > spleen > bone marrow > brain. Peak 5-FC concentrations ranged from 15 μg/g in the brain to 2,000 μg/ml in serum.

The pharmacokinetic pattern of converted 5-FU in tumors was similar to that of 5-FC in mice treated with TAPET-CD/5-FC. The concentration of converted 5-FU reached a peak level of 19 μg/g at 30 min in tumors and declined gradually to 5 μg/g at 270 min after 5-FC injection. Low levels of 5-FU (≤0.2 μg/g) were occasionally detected in a few samples in bone marrow and spleen at one or two time points. An intraperitoneal dose of 5-FU (60 mg/kg) gave an intratumoral peak level of approximately 4.7 μg/g at 30 min after administration.

Antitumor activity of TAPET-CD

Figure 4A shows the antitumor activity of TAPET-CD/5-FC in C38 murine colon tumors. Tumor growth of C38 was not altered by treatment with 5-FC. A significant inhibition (79% at day 40) of tumor growth was obtained in mice that received a single injection of TAPET-CD. In the group treated with TAPET-CD/5-FC, all tumors underwent regression during the first week of treatment, reached their nadir (96% inhibition) on day 26, and then grew slowly, reaching approximately their

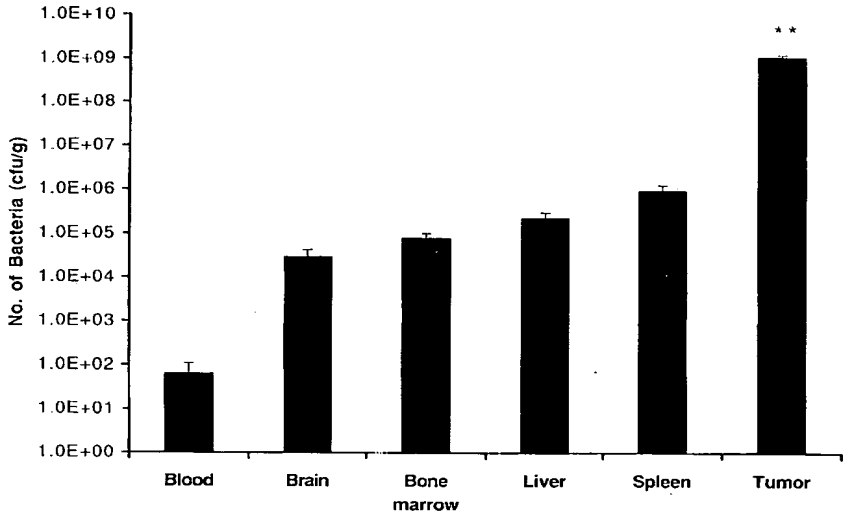


FIG. 1. Tissue distribution of TAPET-CD in mice implanted with B16-F10 melanoma. Mice were administered intravenously with TAPET-CD at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Bacteria content in tissues were evaluated 3 days after bacteria inoculation. **denotes $p < 0.01$ between tumor and normal tissues.

original sizes on day 40. The tumors in the TAPET-CD/5-FC–treated animals were significantly smaller than those in either the 5-FC or TAPET-CD groups from day 26 to the completion of the experiment ($p < 0.01$ between TAPET-CD/5-FC and TAPET-CD on day 40).

As indicated in Figure 4B, WiDr tumors in the PBS control group grew exponentially, increasing in size 10- to 12-fold over the time period examined. 5-FC had little effect on the growth of WiDr tumors in mice (12.5% inhibition on day 47). A single injection of TAPET-CD caused some degree of tumor inhibition (58%) on day 47. The antitumor effect of TAPET-CD/5-FC was not apparent until after 1 week of 5-FC injection. At the completion of the experiment (day 47), profound antitumor effects (88% inhibition) were observed in the group treated with TAPET-CD/5-FC. $p < 0.05$ was obtained for TAPET-CD/5-FC versus TAPET-CD on day 47.

TAPET-CD alone had only modest antitumor effects (38% inhibition at day 54) in LoVo tumors. Pronounced antitumor activity was obtained in mice treated with TAPET-CD/5-FC. The treatment of TAPET-CD/5-FC resulted in an 89% inhibition of tumor growth compared to the control group. No effects on tumor growth were observed in the control group treated with 5-FC alone (Fig. 4C). $p < 0.01$ was obtained for TAPET-CD/5-FC versus TAPET-CD on day 54.

Similar weight loss (10%–15% of original body weight) was observed in mice treated with TAPET-CD alone and TAPET-CD/5-FC. At 300 mg/kg, 5-FC was relatively nontoxic to mice. No animals died in groups treated with TAPET-CD alone or TAPET-CD/5-FC.

5-FU, at 50 mg/kg, given once daily for 5 days, produced significant toxicity when used alone or in combination with TAPET-CD. Weight loss in mice treated with 5-FU was greater than 15% of original body weight. The experiment was terminated 10 days after 5-FU dosing because of severe toxicity. The mortality in both groups treated with 5-FU and TAPET-CD/5-FU was greater than 40%. No additive antitumor activity between 5-FU and TAPET-CD could be observed because of the early termination of the experiment.

DISCUSSION

We have previously demonstrated that the genetically modified *Salmonella typhimurium*, VNP20009, targets and replicates intratumorally in syngeneic tumors and human tumor xenografts in murine models (Pawelek *et al.*, 1997; Zheng *et al.*, 2000). The use of VNP20009 as a delivery system for antitumor agents has numerous advantages for gene therapy. First, amplification within tumors is highly selective, surpassing the specificity observed for tumor-specific antibodies. Second, amplification theoretically requires only one bacterium to seed the tumor site, making delivery highly efficient and increasing the likelihood of reaching metastases that are inaccessible to other therapeutic methods. Third, systemic administration enables accumulation of the therapeutic vector at tumor sites. Fourth, the expression of enzymes or proteins at high levels makes the vector ideal for carrying prodrug-converting enzymes, cytokines, antiangiogenic peptides, antigenic peptides for immunomodulation, or other agents individually or in combination into tumors. Fifth, long-term, sustained expression of antitumor agents within a tumor can be achieved, because of the slow clearance of bacteria from the tumor.

In this study, we have evaluated the tissue distribution of TAPET-CD, *in vivo* CD activity by measuring the conversion of 5-FC to 5-FU, and antitumor activity of TAPET-CD, both alone and with 5-FC. We demonstrated that TAPET-CD preferentially replicates and accumulates in tumors and consistently expresses active CD that converts 5-FC to 5-FU efficiently and

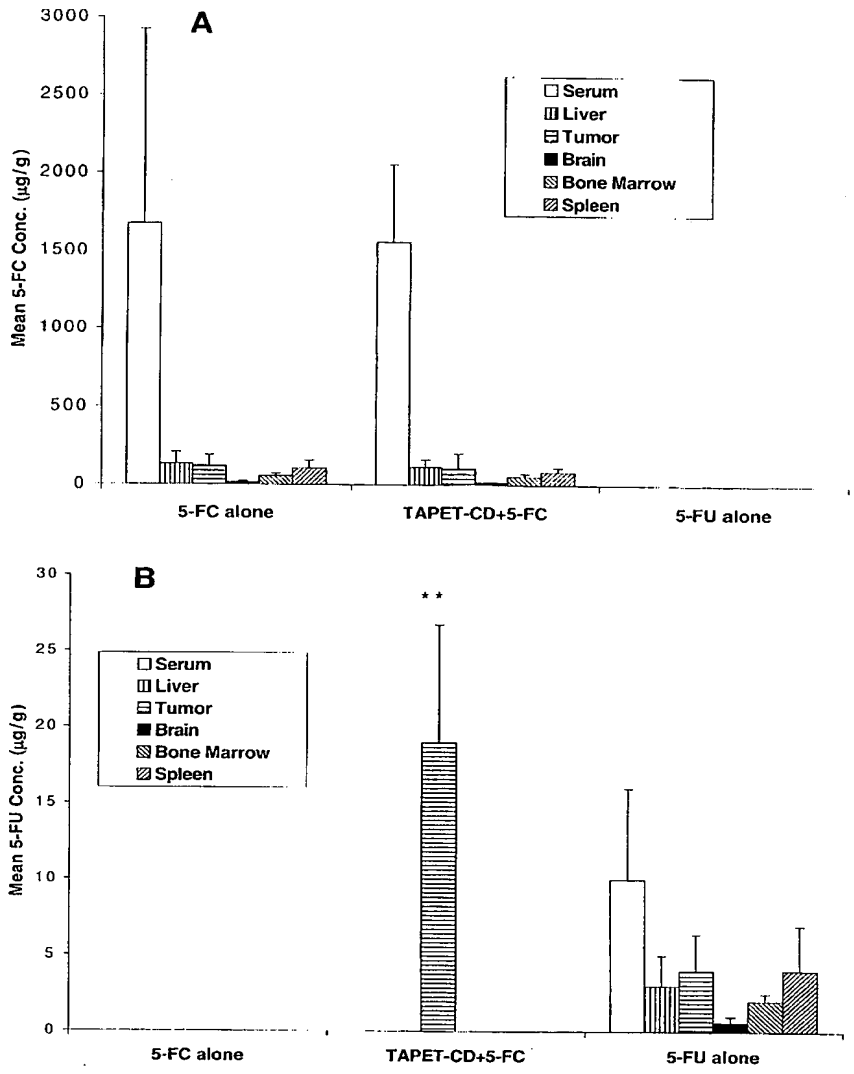


FIG. 2. Tissue distribution of 5-fluorocytosine (5-FC) (A) and 5-fluorouracil (5-FU) (B) in mice implanted with B16-F10 melanoma. Mice were administered intravenously with TAPET-CD at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Tissues were collected 30 min after a single dose of 5-FC (300 mg/kg) or 5-FU (60 mg/kg) was administered intraperitoneally. For 5-FC and 5-FU groups, phosphate-buffered saline (PBS) instead of TAPET-CD was given. Three mice were used for each group and **denotes $p < 0.01$ between groups treated with 5-FU alone and TAPET-CD/5-FC.

locally. Because the amount of TAPET-CD in normal tissues is approximately 1000-fold less than that in tumors, the conversion of 5-FU in normal tissues was undetectable. After a single dose of 300 mg/kg 5-FC, the converted 5-FU in tumors reached the highest level (19 μg/g) at 30 min, then gradually declined at 4.5 hr to 5 μg/g, a level that remains cytotoxic to many tumor cell lines. A minimal or undetectable level of 5-FU was found in normal tissues. In contrast, when given directly to mice, 5-FU distributed relatively randomly in most tissues, with levels of 5-FU in blood, liver, and spleen becoming higher than in tumors. For example, at 60 mg/kg 5-FU, a dose that causes mild toxicity, intratumoral concentrations of 5-FU reached 4.7 μg/g at 30 min and dropped below an effective concentration of 1 μg/g at 90 min. In a cancer patient receiving 300 mg of the antitumor drugs UTF or tegafur, which are derivatives of 5-FU, intratumoral concentrations of 5-FU were approximately 0.18 μg/g at 5.5 hr after drug administration (Arima *et al.*, 1986). TAPET-CD/5-FC clearly produced a higher and more prolonged presence of 5-FU in tumors. Higher intratumoral 5-FU levels could likely produce a higher antitu-

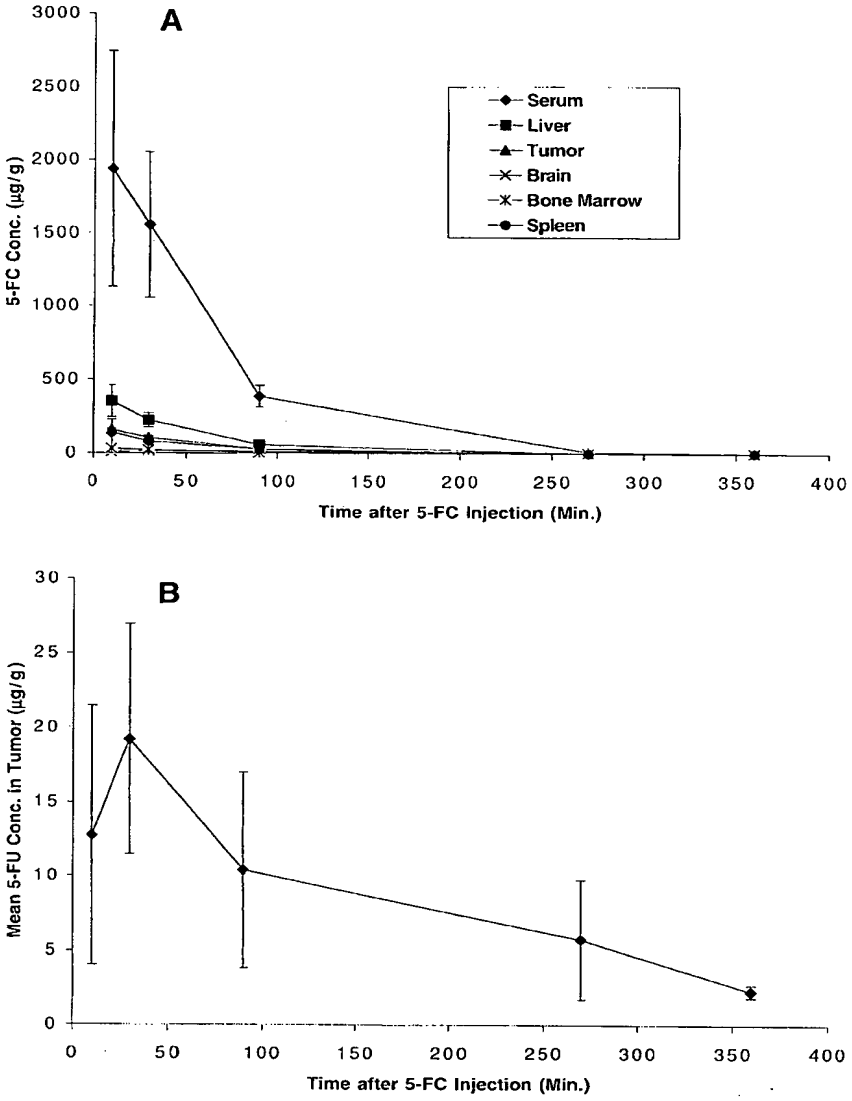
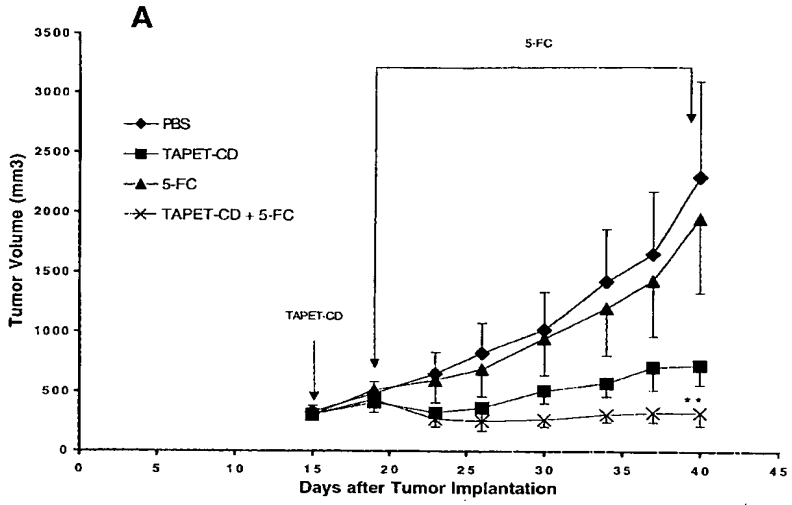


FIG. 3. Pharmacokinetics and tissue distribution of 5-fluorocytosine (5-FC) (**A**) and 5-fluorouracil (5-FU) (**B**) in mice implanted with B16-F10 melanoma. Mice received TAPET-CD intravenously at 1×10^6 colony-forming units (cfu) per mouse 14 days after tumor implantation. Tissues were collected 10 to 360 min after a single dose of 5-FC (300 mg/kg) or 5-FU (60 mg/kg) was administered intraperitoneally to TAPET-CD group or phosphate-buffered saline (PBS)-treated group, respectively. Three mice were used for each group.

mor effect (Presant *et al.*, 1994; Gamelin *et al.*, 1996; Muller *et al.*, 2000).

Using murine and human colon tumor models, we unequivocally demonstrated that TAPET-CD/5-FC produced a superior antitumor effect than either agent acting alone. Tumor regression was observed in some mice receiving the combination regimen. TAPET-CD and CD activity persisted in B16-F10 melanomas for a minimum of 14 days (Zheng *et al.*, 2000). During

FIG. 4. Tumor growth inhibition of C38 murine colon carcinoma (**A**), WiDr human colon carcinoma (**B**), and LoVo human colon carcinoma (**C**) by TAPET-CD and 5-flurocytosine (5-FC). TAPET-CD [1×10^6 colony-forming units (cfu) per mouse] was injected intravenously to mice bearing C38 (day 15), WiDr (day 21), or LoVo (day 20) carcinoma when tumor reached 150–300 mm^3. 5-FC was given intraperitoneally at 300 mg/kg, three times daily, as indicated. Seven to 10 animals were used per group. *denotes $p < 0.05$ and **denotes $p < 0.01$ between TAPET-CD alone and TAPET-CD/5-FC treated group.


A
5-FC
PBS
TAPET-CD
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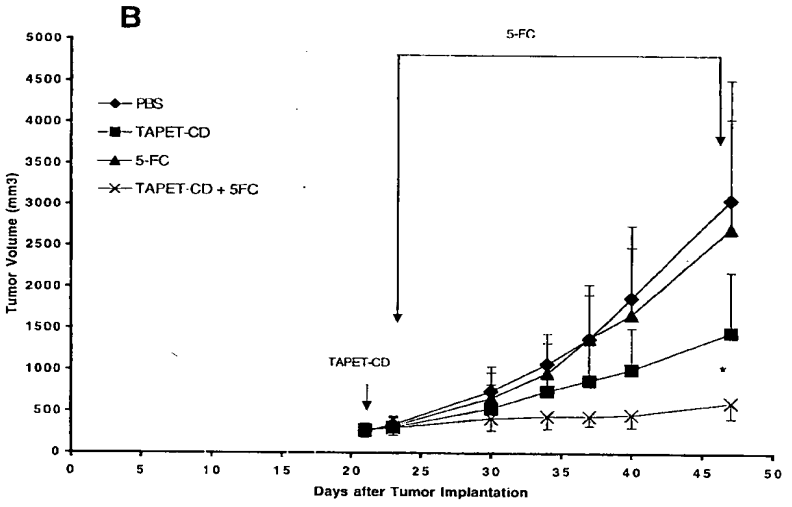
Tumor Volume (mm3)
Days after Tumor Implantation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5-FC
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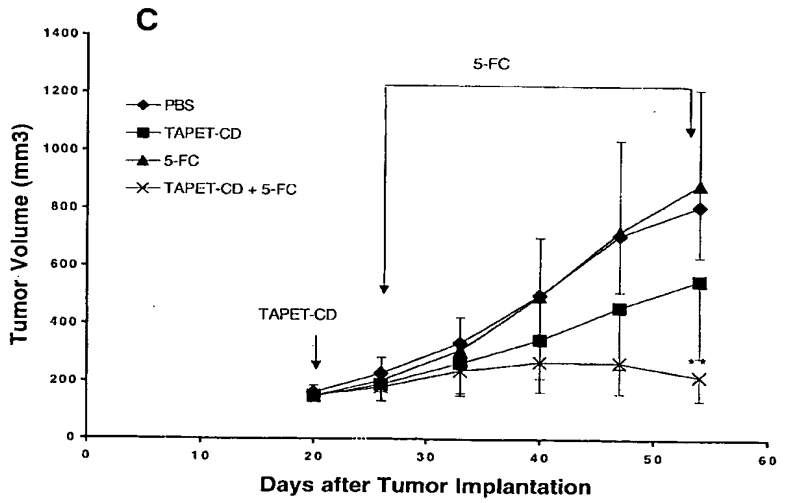
TAPET-CD
Tumor Volume (mm3)
Days after Tumor Implantation


C
5-FC
PBS
TAPET-CD
5-FC
TAPET-CD + 5-FC
TAPET-CD
Tumor Volume (mm3)
Days after Tumor Implantation

the course of the *in vivo* efficacy experiment, we further detected the presence of TAPET-CD in LoVo colon tumors for up to 32 days after bacteria administration (data not shown). It is clear that a single injection will provide CD for an extended period of time. However, 5-FC and 5-FU have short half-lives and repeated dosing of 5FC is needed. 5-FC is used as an antifungal agent in humans and is given orally at 1.3 g/m^2 four times daily. It is conceivable that a prolonged, high intratumoral concentration of 5-FU can be maintained by multiple daily administration of 5-FC. Toxicity of TAPET-CD/5-FC, determined by body weight loss, is similar to that of TAPET-CD or 5-FC alone. Combinations of TAPET-CD and 5-FU at effective doses produced higher toxicity, in terms of body weight loss and mortality, than either agent alone. In addition, 5-FU did not increase the anti-tumor efficacy induced by TAPET-CD. 5-FU is not a potent anticancer agent, and continuous infusion is required in human cancer treatment protocols to optimize clinical outcomes. In the present study, frequent dosing with 5-FC was also required to obtain optimal effects. However, continuous infusion of 5-FU in human cancer patients increases toxicity to normal tissues. We believe that TAPET-CD/5-FC provides a solution for generating therapeutic concentrations of 5-FU locally, while avoiding excessive toxicity in normal tissues.

Although the conversion of intratumoral 5-FC to 5-FU is efficient, it likely could be improved. The AUC of 5-FC and 5-FU is 7290 mg · min per kilogram and 2789 mg · min per kilogram, respectively (data not shown), with approximately 30% of 5-FC converted. The TAPET vector uses CD from *E. coli*, which has a higher Km (Kievit *et al.*, 1999) than the fungal CD. It is conceivable that a fungal CD expressed in a TAPET vector could achieve a higher conversion rate and, subsequently, a higher concentration of 5-FU, resulting in a higher antitumor activity. However, increasing CD activity in the TAPET vector could also increase toxicity of the TAPET-CD/5-FC system. A low level of 5-FU was occasionally detected in bone marrow and spleen of mice receiving both TAPET-CD and 5-FC.

The successful use of TAPET-CD/5-FC also suggests the possibility of using attenuated *Salmonella* strains as tumor-specific delivery systems for other protein-based antitumor agents, such as cytokines, antiangiogenic proteins, and prodrug-converting enzymes. *Salmonella* strains have been shown to express a broad spectrum of therapeutic proteins, such as interleukin (IL)-1, IL-2, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α (Saltzman *et al.*, 1996, 1997; Lin, 1999; Yuhua *et al.*, 2001). TAPET-CD is currently being evaluated in cancer patients (Cunningham and Nemunaitis, 2001) as a potential anticancer agent. We are also currently evaluating the expression of various therapeutic proteins in TAPET vectors and the antitumor activities of these therapeutic vectors.

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Pilot trial of genetically modified, attenuated Salmonella expressing the *E. coli* cytosine deaminase gene in refractory cancer patients

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We performed a pilot trial in refractory cancer patients to investigate the feasibility of intratumoral injection of *TAPET-CD*, an attenuated Salmonella bacterium expressing the *E. coli* cytosine deaminase gene. A total of three patients received three dose levels of *TAPET-CD* (3×10^6–3×10^7 CFU/m^2) via intratumoral injection once every 28 days as long as progression of disease or intolerable toxicity was not observed. From days 4 to 14 of each 28 day cycle, patients also received 5-fluorocytosine (5-FC) at a dose of 100 mg/kg/day p.o. divided three times daily. Six cycles of treatment were administered. No significant adverse events clearly attributable to *TAPET-CD* were demonstrated. Two patients had intratumor evidence of bacterial colonization with *TAPET-CD*, which persisted for at least 15 days after initial injection. Conversion of 5-FC to 5-fluorouracil (5-FU) as a result of cytosine deaminase expression was demonstrated in these two patients. The tumor to plasma ratio of 5-FU for these two colonized patients was 3.0, demonstrating significantly increased levels of 5-FU at the site of *TAPET-CD* colonization and insignificant systemic spread of the bacteria. In contrast, the tumor to plasma ratio of 5-FU of the patient who did not show colonization of *TAPET-CD* was less than 1.0. These results support the principle that a Salmonella bacterium can be utilized as a delivery vehicle of the cytosine deaminase gene to malignant tissue and that the delivered gene is functional (i.e. able to convert 5-FC to 5-FU) at doses at or below 3×10^7 CFU/m^2.

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Several preclinical studies have shown that systemically administered bacteria will accumulate preferentially in the extracellular compartment of tumors. Conditions present in tumors such as hypoxia, presence of cellular debris from necrosis, high interstitial pressure that impede entry of antibodies, or the expression of immunosuppressive substances probably favor the selective growth of bacteria in the tumor microenvironment. Preclinical studies involving systemic administration of various attenuated and nonattenuated bacteria also indicate that bacterial colonization of malignant tissue can be associated with tumor necrosis and growth inhibition.[1–4] In the medical literature, spontaneous infection of human tumors has been reported.[5–13] Based on these findings, a small number of advanced cancer patients were treated systemically (or by direct tumor injection) with non-toxin-producing strains of Clostridia in the 1960s, but this approach was abandoned due to excessive toxicity and minimal antitumor effect.

Spontaneous infection of human tumors with *Salmonella* species has been well-documented,[9–13] and for potential systemic administration as a cancer therapeutic, *Salmonella* offer several advantages over other bacterial species, including the relative ease of attenuation, sensitivity to antibiotics, and ability to grow in both hypoxic and well-oxygenated environments. Recently, a *S. typhimurium* species was genetically attenuated by deleting both the *purI* and *msbB* genes (VNP20009).[14–16] The *purI* deletion creates a requirement for an external source of adenine, thus limiting growth to areas that have substantial cell turnover, death, and cellular debris (i.e. sites of malignant growth).[17] The deletion of the *msbB* gene, which is required for synthesis of lipid A, was engineered to reduce toxicity associated with induction of tumor necrosis factor-α (TNF-α).[18] VNP20009 has been

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administered intravenously to several species with minimal to modest reversible toxicity.[14,15] Despite its substantial attenuation, VNP20009 effectively colonized syngeneic, spontaneous, and human tumors in mice, formed high tumor-to-normal tissue ratios (range from 250:1 to 25,000:1 compared to liver), and inhibited tumor growth.[14,15,19–21]

Recently, a phase I clinical trial involving 25 patients, who received intravenous infusion of VNP20009, defined a maximum tolerated dose of 3×10^8 CFU/m^2.[22] Colonization of tumor was detected in three patients however, objective tumor regression was not observed. Dose-related increases in circulation of interleukin 1 beta, tumor necrosis factor alpha, and interleukin 6 appeared to be associated with clinical toxicity, that is, hypotension, fever, thrombocytopenia, anemia, diarrhea, nausea, and vomiting.[22,23] A second study involved direct intratumoral administration of VNP20009. Approximately 2 weeks following the administration of VNP20009, VNP20009 could be detected in biopsies of most injected lesions, and in some necrosis, inflammation, and/or regression was observed. The intratumoral administration was associated with minimal to modest systemic and local toxicity at doses between 3×10^6 and 3×10^8/m^2 (unpublished data).

In an effort to increase the therapeutic effectiveness of VNP20009 the cytosine deaminase (*CD*) gene from *E. coli* was genetically inserted into the chromosome of VNP20009 at the ΔmsbB locus to create the strain *TAPET-CD*.[19,20,24] *CD* is an enzyme found in both bacteria and fungi, but not in mammalian cells. It converts 5-fluorocytosine (5-FC), an antifungal agent with limited systemic toxicity, to 5-fluorouracil (5-FU).[25] 5-FU is a cytotoxic antimetabolite commonly used to treat colorectal, gastric, head and neck, pancreatic, and breast cancers.[26] Clinical data suggest that the antitumor activity of 5-FU is directly related to both the duration of drug exposure and its concentration in the tumor. Administration of *TAPET-CD* and 5-FC in murine tumor models produced selective high 5-FU concentrations in malignant tissue, which correlated with tumor colonization by *TAPET-CD*.[24] Intratumor concentration of 5-FU following intravenous infusion at optimal doses (60 mg/kg), associated with anticancer activity, failed to achieve similar intratumoral concentration of 5-FU compared to intravenous *TAPET-CD*/5-FC.[24,27] *TAPET-CD* and 5-FC also demonstrated significantly enhanced antitumor activity without additional toxicity in animal models in comparison to *TAPET-CD* alone.[19,20] Others have also confirmed significant improvement in antitumor activity of the encoded *CD* gene compared to the respective parent wild-type bacteria with different attenuated bacterial species.[28]

Based on clinical data generated with the parent VNP20009 at doses up to 3×10^8 CFU/m^2 and efficacy demonstrated in mice with *TAPET-CD*/5-FC, we initiated a pilot trial of intratumoral injection of *TAPET-CD* followed by oral 5-FC in patients with refractory cancer. The objectives of this trial were to determine the MTD of *TAPET-CD* in combination with 5-FC; to quantitate *TAPET-CD* colonization in tumor tissue; to determine the blood pharmacokinetics of *TAPET-CD* following intratumoral injection; and to determine the blood and intratumoral concentrations of 5-FC and 5-FU. Although the trial was discontinued for nonmedical issues after three patients were accrued, proof of concept was demonstrated by the intratumor conversion of 5-FC to 5-FU. No significant toxicity was observed.

Methods

Study population

Patients were eligible for this study if they had advanced and/or metastatic, histologically documented solid tumors that were considered sensitive to 5-FU and if their disease was no longer responding to available conventional modalities or treatment (failed any known standard curative or effective therapy for that disease). The specific tumor to be injected with *TAPET-CD* had to be at least 1.5 cm in diameter, cutaneous or subcutaneous, if in lymph nodes easily accessible near the body surface, or on a mucosal surface or in soft tissues of the extremities. All lesions had to be accessible to surgical incision and drainage. Other eligibility criteria included ECOG performance status of 0 or 1, age $\geq$18 years, life expectancy of $\geq$3 months, granulocyte count $\geq$2,000/mm^3, platelets $\geq$100,000/mm^3, serum creatinine $\leq$2.0 mg/dl, bilirubin, and alkaline phosphatases $\leq 1.5\times$ the upper limit of normal, ALT and AST $\leq 1.5\times$ the upper limit of normal, hematocrit $\geq$30%, maximum daily temperature of $\leq$38.0°C, and consent for study participation on an IRB-approved informed consent form. Women and men of reproductive potential were required to use a reliable form of contraception.

Patients were excluded from the study if their lesions were in a location that was unable to accommodate swelling or inflammation, in the central nervous system, or in the retroperitoneum or mediastinum. Other exclusion criteria included patients with metastatic lesions that would not be treated as part of the protocol and that would require palliation with surgery or other modalities in $<$28 days, an artificial implant that could not be easily removed, known cardiac valvular disease or arterial aneurysms, known clinically significant atherosclerotic disease, gallstones or urinary tract stones, an active infection of any kind, any known hypocoagulation disorder, documented immunodeficiency, evidence of chronic active Hepatitis B, active brain metastases, or hypersensitivity to quinoline or cephalosporin antibiotics. Patients who were currently using antibiotics, or who required chronic steroid or immunosuppressive medications were also ineligible for the study. Commercial food handlers, day-care workers, health-care workers or those unable to avoid close personal contact with severely immunosuppressed individuals were not allowed to participate in the study. This study was approved by the local institutional review board and all patient participants were required to sign consent before participation.

Design

This was an open-label, phase 1, dose-escalation study. On day 1, prior to treatment with *TAPET-CD*, patients were administered 500 cm^3 of D5W with half-normal saline over 2–3 hours to ensure that they were well hydrated. At 2 hours before receiving *TAPET-CD* they started a regimen of 650 mg of acetaminophen orally every 4 hours and 50 mg of indomethacin every 8 hours for 24 hours and then as needed for temperatures $\geq 39°C$. While on indomethacin they also received 50 mg of ranitidine IV every 8 hours or 200 μg of cytotec each day. Patients received direct intratumoral injections of *TAPET-CD* on day 1 with a total injection volume of approximately 10% of the tumor volume. One patient per dose level was planned unless a grade 2 toxicity attributable to study treatment developed. The first patient was treated at a dose level of 3.0×10^6 CFU/m^2 of *TAPET-CD*, the second patient at 1×10^7 CFU/m^2, and the third patient at 3.0×10^7 CFU/m^2. If all toxicities of *TAPET-CD* were resolved to $\leq$grade 1 (with the exception of transaminases and hematologic abnormalities, which had to be $\leq$grade 2), then on day 4, oral 5-FC (100 mg/kg/day divided thrice daily) was begun and continued for 14 days. This cycle was repeated on days 29 and 58 in eligible patients. Patients were monitored by CT scan after every cycle and were eligible to begin the next cycle if the injected lesion showed no signs of progression and they continued to meet all other eligibility criteria.

Monitoring of patients

Chest X-ray, physical examination, complete history, performance status, tumor staging, ultrasound of the abdomen, blood cultures, urine and stool cultures, vital signs, urinalysis, CBC and platelets, PT/PTT, liver function tests (LFTs), serum chemistry, pregnancy test, HIV test, Hepatitis B surface antigen test, EKG, and antibody response were all performed prior to treatment with *TAPET-CD*.

During, and following, the dose of *TAPET-CD*, vital signs and pulse oxygen saturation were obtained every 15 minutes for 90 minutes, then every 30 minutes x 4, then every 2 hours x 4, then every 4 hours through the first 24 hours or until discharge from the outpatient area (at least 8 hours after treatment). On days 2 and 4 patients were monitored with vital signs, performance status, CBC with platelets, serum chemistries, LFTs, urinalysis, blood cultures, and urine and stool cultures. Blood samples to perform PK analysis of *TAPET-CD* were drawn on day 1 and for 5-FC and 5-FU on day 4. Patients returned to the outpatient clinic on days 8 and 15 and the same procedures were performed as on day 4, with the exception that no urine and stool cultures were taken. Tumor samples, to verify *TAPET-CD* colonization and to measure intratumoral 5-FC and 5-FU concentrations were obtained on day 8 and, in selected patients, between days 15 and 17 to confirm the persistence of high levels of bacteria in the tumor and to provide a second measurement of 5-FC and 5-FU intratumoral concentrations. On day 11, CBC and platelets were drawn. On day 18, CBC and platelets were also drawn and a blood for PK evaluation for 5-FC and 5-FU was performed.

The NCI Common Toxicity Criteria were used to grade toxicity. The dose-limiting toxicities for *TAPET-CD* were defined as follows: $\geq$grade 3 toxicity, hypotension requiring any vasopressor support, $\geq$grade 2 allergic toxicity, grade 2 fever for more than 6 hours per day that persisted for >1 week and grade 2 constitutional symptoms which persisted greater than 1 week or were poorly controlled with supportive medication. Any inflammation induced in the brain, anterior chamber of the eye, or joints or grade 2 toxicities involving the central nervous system, cardiac, pulmonary, and renal function not resolving within 48 hours were also considered dose-limiting toxicities.

TAPET-CD

The *CD* gene was cloned from *E. coli* by PCR using primers based on the complete sequence for the *E. coli* cytosine deaminase[27]: forward: 5′-GAT CAT GCA TGT GGA GGC TAA CAG T-3′ and reverse 5′-CTA GAT GCA TCA GAC AGC CGC TGC GAA GGC-3′. The *CD* gene was further subcloned into the vector pTrc99a (Pharmacia -Upjohn) and sequenced to verify. A vector capable of undergoing homologous recombination with the ΔmsbB gene in the chromosome of strain VNP20009[16] was created in the suicide vector pCVD442[29] to create pCVD442-*msbB*. A DNA fragment from the pTrc99a-CD vector above was generated by partial digestion with *SspI* and subcloned into the vector pCVD442-*msbB* to create pCVD442-CD. The *CD* gene was than transduced and integrated into the chromosome of VNP20009 by the method of Donnenberg and Kaper,[29] to create the strain *TAPET-CD*. A Research Cell Bank (RCB) and Master Cell Bank (MCB) were created and the location and sequence of the inserted *CD* gene at the ΔmsbB locus was confirmed in the MCB by sequence analysis. The clinical lot of *TAPET-CD* material was produced under GMP conditions by Cambrex Bioscience (formerly Marathon Pharmaceuticals, Hopkinton, MA).

Quantitation of TAPET-CD *accumulation and 5-FC/5-FU conversion in tissues*

Tumor tissue was excised under sterile conditions and homogenized in PBS. Bacteria were quantitated by plating serial dilutions of the homogenates onto rich media, incubating overnight at 37°C, and counting bacterial colonies. A single colony arising from a single plated bacteria which was visibly detected in the culture media was considered a positive colony. The conversion of 5-FC to 5-FU in tissue was determined by high-performance liquid chromatography (HPLC) analysis based on the maximum absorbance of 5-FU at 266 nm and 5-FC at 280 nm (Phenomenex Prodigy ODS (3) column). The detection limits for 5-FC and 5-FU were 0.5 and 0.2 $\mu g/g$ respectively.

Preparation and administration of TAPET-CD

TAPET-CD was stored at −80°C in 15% glycerol. Each vial contained 1.2 ml of *TAPET-CD* bacteria at approximately 3×10^9 CFU/ml. Vials of *TAPET-CD* were thawed at room temperature for 15–20 minutes. The stock *TAPET-CD* was serially diluted into sterile normal preservative free saline and the appropriate volume of diluted *TAPET-CD* was divided equally between two and four injections and delivered using a 23–27 gauge needle. Prior to injection, negative pressure was created to avoid injection into the circulation. Each syringe was used to make a separate skin puncture, at approximately 60–80% of the distance from the tumor center out to the tumor periphery. The skin punctures were evenly distributed around the perimeter of the tumor and over its surface. With each skin puncture, six to eight needle tracks were made emanating out radially from the puncture site and the bacteria was distributed uniformly along the needle track by gradually depressing the syringe plunger during withdrawals of the needle. Most of the bacterial dose was delivered to the tumor periphery at the interface of the tumor and normal tissue.

Results

Patient population

Three patients received injections of *TAPET-CD* from 1/29/02 to 5/16/02 in the Mary Crowley Medical Research Center, Dallas, Texas. A fourth patient signed consent but withdrew during screening and did not receive treatment. Results are reported on the three patients who received treatment. Demographics of these three patients are listed in Table 1. Dosing of *TAPET-CD* is listed in Table 2.

Adverse events

Toxicity felt to be related to *TAPET-CD* is listed in Table 3. One grade 3 toxicity was observed in patient 001, 4 days after initiation of cycle 3. She developed symptoms consistent with either esophageal spasm or coronary vessel spasm. Transient S-T segment EKG changes in association with chest pain were observed during a follow-up clinic visit. Pain and EKG changes were relieved with sublingual nitroglycerin and the patient was admitted for observation and further work-up. Cardiac enzymes were not elevated, and no evidence of obstruction by coronary catheterization was observed, no arrhythmias were observed during cardiac monitoring. The patient was released without further treatment and no recurrent events have occurred following release. Other AEs, defined in Table 3, were likely related to reactivity to colonization of bacteria (i.e. fever and fatigue).

TAPET-CD *tissue colonization and blood levels*

Tissue colonization for each patient is shown in Table 4. Patients 001 and 003 demonstrated colonization of *TAPET-CD* within tumor tissue. Colonization was seen in both course 1 and course 2 in patient 001. In both patients, tissue colonization persisted in tumor tissue for at least 15 days. The tumor tissue from patient 001 was contaminated with oral flora (note that this was a tumor of the oral cavity), requiring *TAPET-CD* to be isolated using selective media. The use of selective media is known to reduce the recovery of *TAPET-CD* organisms;

Table 1 Demographics

Patient number	Sex	Patient age (years)	ECOG	Site of disease to be injected	Histologic tumor type/primary	Prior treatment
001	F	89	1	Hard palate (4.6 cm × 4.6 cm)	Squamous cell (verrucous)/head and neck	Surgery, radiotherapy
002	M	54	1	Left anterior neck (2.2 cm × 1.6 cm)	Adenocarcinoma/ esophagus	5-FU, taxol, ifosfamide, cisplatin, CPT-11, xeloda, radiotherapy
003	M	50	1	Right scapula (3.0 cm × 3.5 cm)	Adenocarcinoma/ esophagus	Esophagiogastrectomy, cisplatin, 5-FU, carboplatin, radiotherapy

Table 2 Summary of treatment

	TAPET-CD			5-FC	
Patient number	Dose level (CFU/m^2)	Dose (CFU/inj)	No. of Injections (per cycle)	Dose (mg/kg/day)	No. of days dosed (per cycle)
001	3.0×10^6	4.62×10^6	12 (4, 4, & 4)	100	32 (14, 14, & 4)
002	1.0×10^7	3.80×10^7	4 (2 & 2)	100	28 (14 & 14)
003	3.0×10^7	5.04×10^7	4 (4)	100	14 (14)

Table 3 Adverse events (Grade 2 and Higher)

Patient number	Adverse event	Grade	Related to study drug
001	Coronary vasospasm (SAE)	3	Possibly
	Tumor necrosis	2	Probably
	Weakness	2	Possibly
	Insomnia	2	Possibly
002	Fever	2	Probably
003	Pain	3	Probably

Table 4 Tissue colonization of *TAPET-CD*

Patient	Course	Day	CFU/g
001[a]	1	8	3.4×10^3
		15	84
	2	8	1.4×10^3
		15	850
002	1	8	67
		15	Neg
	2	8	Neg
		15	Neg
003	1	8	2.6×10^6
		15	2.6×10^5

Neg=Negative, no detectable viable bacteria in tissue.
[a]Patient 001 tissues samples from mouth, highly contaminated with multiple flora. *TAPET-CD* identified by selective media causing significant reduction in recovery.

however, even under these conditions significant levels of *TAPET-CD* could be isolated from the tumor tissue of patient 001.

The concentration of *TAPET-CD* in the blood was also monitored after injection. At early time points (30 minutes to 1 hour postinjection) the blood levels were detectable but below the level of accurate quantitation (mean values ranged from 1.8 to 3.0 CFU/ml). All later time points *TAPET-CD* values were below detection levels. The pattern of distribution of *TAPET-CD* throughout the tumor tissue was not assessed.

5-FC and 5-FU tissue and plasma levels

Plasma levels of 5-FC and 5-FU are provided in Table 5. Steady-state concentration (Css) in plasma of 5-FC was achieved by day 8 and ranged from 923 to 1231 μM. The level of 5-FU in plasma also reached steady-state condition by day 8, and ranged from 0.39 to 0.62 μM.

A summary of tissue levels of 5-FC and 5-FU are shown in Table 6 for each patient. The concentration of 5-FU in tumor tissue from patients 001 and 003 was more than 5 times greater than the concentration of 5-FU in the tumor of patient 002, who did not show evidence of tumor colonization (Table 4). The tumor to plasma ratio for 5-FC of each patient was approximately 0.6, as would be expected for small molecule drugs (Table 7). The tumor to plasma ratio of 5-FU for patients 001 and 003, both of

Table 5 Plasma levels of 5-FC and 5-FU

Patient	Course	Day	Protocol time point (hours)	5-FC (μM)	5-FU (μM)
001	1	4	Pre	ND	ND
			1	49.6	0.041
			2	185.1	0.126
			4	312.1	0.203
			8	341.3	0.178
		8	Baseline	941.6	0.416
			1	926.6	0.494
			2	998.7	0.633
			4	1150.8	0.532
			8	843.6	0.514
		15	1	1223.5	0.869
	2	4	Pre	ND	ND
			1	9.8	0.009
			2	266.3	0.192
			4	469.8	0.418
			8	333.2	0.242
		8	Baseline	1056.9	0.534
			1	940.7	0.618
			2	1071.8	0.385
			4	1170.4	0.588
			8	853.5	0.408
		15	1	1134.3	0.577
002	1	4	Pre	ND	ND
			1	111.5	0.175
			2	189.2	0.230
			4	396.2	0.473
			8	208.5	0.266
		8	Baseline	348.5	0.431
			1	321.5	0.406
			2	282.3	0.265
			4	537.7	0.520
			8	351.5	0.468
		15	1	400.8	0.211
	2	4	Pre	ND	ND
			1	150.0	0.203
			2	159.2	0.182
			4	259.2	0.343
			8	273.8	0.292
		8	Baseline	305.8	0.239
			1	376.9	0.295
			2	474.6	0.328
			4	433.8	0.490
			8	372.3	0.233
		15	1	503.1	0.347
003	1	8	1	355.4	0.378
			2	375.0	0.347
			4	280.8	0.386
			8	153.8	0.223
		15	1	296.7	0.372

ND=not detected.

whom had high levels of tumor colonization (Table 4), was approximately 3.0. The tumor to plasma ratio of 5-FU for patient 002 who did not show colonization of *TAPET-CD* bacterium was not different from that observed with 5-FC. Tumor to plasma ratios are compared graphically in Figure 1.

Response

Patient 001 was removed from trial during cycle 3 with stable disease due to the grade 3 toxic event previously described. Necrosis of the patient's fungating oral cavity mass was observed during the first week following each injection; however, despite external evidence of regression and improved subjective air movement with the patient's oral cavity, radiologic assessment of the overall tumor mass did not identify objective tumor regression. Patient 002 was removed after 2 cycles due to progressive disease of noninjected disease sites. The injected site achieved a stable disease response. Patient 003 was removed after completing one cycle due to progressive disease of noninjected disease sites. The injected site achieved a stable disease response. Patient 001 is still alive at 327 days and patient 002 is still alive at 208 days following treatment. Patient 003 passed away of progressive disease from noninjected sites 117 days after treatment.

Table 6 Tissue levels of 5-FC and 5-FU

Patient	Course	Day	5-FC (μM)	5-FU (μM)
001	1	8	418.5	1.064
		15	558.3	1.117
	2	8	654.3	3.072
		15	868.8	1.403
002	1	8	270.9	0.200
		15	195.2	<0.215
	2	8	233.1	0.183
		15	316.4	<0.315
003		8	209.6	0.885
		15	212.2	0.367

Discussion

Results observed in this small pilot trial involving three refractory cancer patients confirm data from preclinical studies that demonstrate selective colonization of *TAPET-CD* in malignant tissue following intratumoral injection. Moreover, conversion of 5-FC to 5-FU was clearly demonstrated in both of the patients with intratumor colonization, indicating that *TAPET-CD* produced functional levels of the cytosine deaminase enzyme within the tumor. The length of the duration in which *TAPET-CD* could be detected in the two patients demonstrating initial colonization was 15 days. We did not measure for the presence of *TAPET-CD* beyond 15 days. Potential colonization of surrounding normal tissue was also not evaluated. However, overall treatment with both the *TAPET-CD* and 5-FC was well tolerated. The clinical response of the three patients treated in this trial was not impressive, although the first patient, who demonstrated the highest concentration of 5-FU within malignant tissue, did maintain stable disease for more than 3 months and is still alive more than 1 year later.

The trial was discontinued prematurely, and a maximum tolerable dose (MTD) was not reached for either the *TAPET-CD* or the 5-FC. The maximum dose of *TAPET-CD* administered was 3×10^7 CFU/m^2, which is below a likely MTD with intratumoral injection. Previous clinical trials with VNP20009 revealed that the MTD via intravenous infusion was 3×10^8 CFU/m^2, and doses of at least this magnitude were administered in the intratumoral study. Higher levels of colonization and greater production of the CD enzyme within tumors could be expected with higher *TAPET-CD* doses. In combination with higher doses of 5-FC, increased colonization of tumor could result in much higher intratumoral 5-FU levels and thus greater antitumor efficacy. Nevertheless, even at the sub-MTD doses administered in this trial, cytotoxic levels of 5-FU were achieved within the colonized tumors.

The current study demonstrated the important proof of concept that bacteria could produce clinically relevant concentrations of functional CD enzyme within tumor. Based on studies conducted with the parent VNP20009, it would appear that VNP20009 and *TAPET-CD* do not

Table 7 Mean steady-state concentration of 5-FC and 5-FU in plasma and tissue (Days 8–15)

		5-FC			5-FU		
Patient	Course	Plasma (μM)	Tissue (μM)	Ratio (tissue/plasma)	Plasma (μM)	Tissue (μM)	Ratio (tissue to plasma)
001	1	1014.2	488.4	0.48	0.58	1.09	1.89
	2	1037.9	761.2	0.73	0.52	2.24	4.32
002	1	373.7	233.0	0.62	0.38	0.2	0.52
	2	411.1	242.8	0.59	0.32	0.2	0.62
003	1	292.4	210.9	0.72	0.34	0.63	1.83

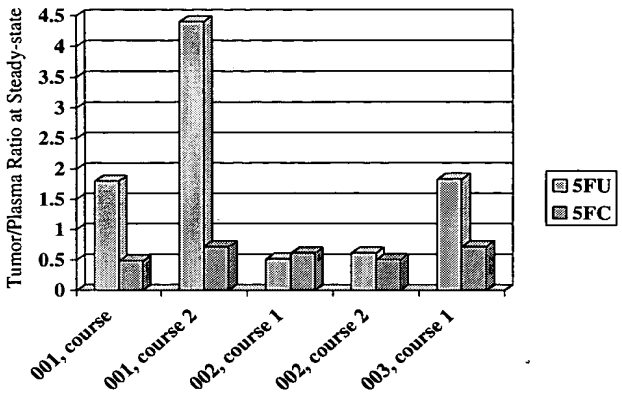

Figure 1 Tissue to plasma ratio's at steady state of 5-FC and 5-FU are compared for each study patient. Biopsy material revealed no evidence of colonization in patient 002, whereas evidence of *TAPET-CD* intratumor colonization of patients 001 and 003 was observed.

colonize human tumors to the same degree as observed in murine models. Although the difference in colonization is most notable following i.v. administration, it is clear that colonization by the intratumoral route could also be improved. Second-generation vectors with improved characteristics for colonization of tumors in patients are under development.

Although ideal development of such bacterial vectors is preferably through intravenous administration, direct tumor injection can be beneficial in various clinical settings. For example, a supra-additive interaction between radiation therapy and *msbB*$^-$ auxotrophic (*purI*) Salmonella has already been shown in the Cloudman S91 xenograft.[30] Supplementation with target selective production of the radiosensitizer, 5-FU, is expected to further enhance this interaction.[31,32] Insofar as attenuated Salmonella colonizes tumor extracellular compartments rather than tumor cell cytoplasm, use of this vector may resolve the problem of preferential "suicide" transduced-cell cytotoxicity (as compared to bystander cells) decreasing bystander effect and, consequently, antitumor efficacy.[33] In addition, the possibility of a CD/5-FC-induced immune mechanism mediating a systemic cytotoxic effect has been postulated but not fully delineated.[34,35] It is unknown to what degree each individual activity with respect to the peak concentration of intratumor 5-FU, the prolonged intratumor presence of 5-FU, or the oncolytic activity of the delivery vehicle will play in determining overall antitumor effect.

Alternative biologic approaches utilizing replicating infectious organisms such as tumor-selective viral particles are also being explored as possible delivery vehicles for the cytosine deaminase gene.[36–38] Others have also shown 5-FC conversion to 5-FU in patients receiving cytosine deaminase gene delivered via nonreplicating adenoviral vectors.[38,39] These preliminary results support future investigation of *TAPET-CD* at higher doses via intratumoral injection. However, since local regional therapeutics have limited utility in oncology a second-generation *Salmonella* vector expressing CD is undergoing preclinical development for potential intravenous injection.

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